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REMARKS

Claims 1-9 and 12-25 are pending. Claims 1-6 and 12-16 are withdrawn from consideration, and claims 7-9 and 17-25 are rejected. Claims 12-16 are cancelled, and claims 1, 7 and 17 are amended herein. Support for the present claim amendments can be found throughout the specification and claims as originally filed, for example, at page 2, first (non-full) paragraph; and page 4, last paragraph.

The amendments are made solely to promote prosecution without prejudice or disclaimer of any previously claimed subject matter. With respect to all amendments, the Applicant has not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the Patent Office. The Applicant expressly reserves the right to pursue prosecution of any presently excluded subject matter or claim embodiments in one or more future continuation and/or divisional application(s).

The Applicant has carefully considered the points raised in the Office Action and believe that the Office's concerns have been addressed as described herein, thereby placing this case into condition for allowance.

Rejections under 35 U.S.C. § 112, first paragraph

Written Description

Claims 7-9 and 17-25 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Applicant respectfully traverses this ground for rejection.

The Office asserts that the Applicant "does not disclose what this CIP fragment is." In response, the Applicant asserts that the intended meaning of the terms "CIP" or "CIP fragment" are set forth in the specification at page 4, third paragraph. Although in abbreviated format, the

specification indicates that the terms "CIP" or "CIP fragment" refer to a contiguous portion of human PTH having an amino acid sequence set forth in SEQ ID NO:3 (PTH_{1-84}), wherein the CIP has the following characteristics: a) the N-terminal amino acid residue of the CIP starts at any position spanning from position 2 through position 34 of the PTH_{1-84}; and b) the C-terminal amino acid residue of the CIP ends at position 84 of the PTH_{1-84}. This description includes and refers to a set of peptides as representative of a CIP fragment, such as PTH_{2-84}, PTH_{3-84}, PTH_{4-84}, PTH_{5-84}, PTH_{6-84}, PTH_{7-84}, PTH_{8-84}, PTH_{9-84}, PTH_{10-84} . . . PTH_{28-84}, PTH_{29-84}, PTH_{30-84}, PTH_{31-84}, PTH_{32-84}, PTH_{33-84}, up to and including PTH_{34-84}. In essence, the term "CIP" or "CIP fragment," as used in the present application means the same thing as if the Applicant had listed out each individual CIP peptide between the listed boundaries. This set of polypeptides is small, clear and has definite boundaries.

Nevertheless, the present claims have been limited to the measurement of a specific polypeptide comprising PTH_{7-84}. This polypeptide comprises a PTH antagonist and falls within the small and defined set of CIP/CIP fragments described above. Moreover, further support for this specific polypeptide can be found in the reference to LePage, R., et al. (*Clin. Chem.* (1998) 44:805-10) in the specification at page 2. This publication was incorporated by reference in the present application and discusses the interference of synthetic PTH_{7-84} in assays for whole PTH and hypothesizes about its physiological activity. It was later verified that PTH_{7-84}, as a PTH antagonist, effects internalization, and ultimately leads to a down regulation, of the 1-84 PTH/PTHrp receptor, without concomitant activation. *See* Sneddon et al., *J. Biol. Chem.* (2003) 278(44):43787-96 (attached hereto at **Exhibit A**). In addition, PTH_{7-84} generally binds and activates a C-terminal receptor that is *independent* of the 1-84 PTH/PTHrp receptor. *See* P. Divieti, et al., *Endocrinology* (2002) 143(1):171-6 (attached hereto at **Exhibit B**).

Accordingly, the Applicant respectfully submits that the written description requirement has been met, especially in view of the present claim amendments. A specific CIP/CIP fragment that is supported in the application, as filed, is identified in the present methods. Withdrawal of this rejection is respectfully requested.

sd-188856

Enablement

Claims 7-9 and 17-25 were rejected under 35 U.S.C. § 112, first paragraph, for allegedly not enabling any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with the claims. The Applicant respectfully traverses this rejection.

In this rejection the Office is again concerned that "[t]he disclosure fails to teach what the cyclase inhibiting parathyroid hormone fragment is." Office Action, page 4, first full paragraph. However, the Office further explains that "[b]ecause the disclosure fails to teach what the fragment is, one of ordinary skill in the art would not be able to generate an antibody for the fragment without undue experimentation and thus one would have a low level of predictability in the art." *Id.* (in the second full paragraph). The Office is additionally concerned that the description supposedly provides no working examples. *Id.* Finally, the Office indicates that since one of skill in the art purportedly does not know what the CIP fragment is, then one could not generate an antibody for the peptide sequence in CIP without undue experimentation. *Id.*

The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. Time and difficulty are not determinative of undue experimentation if the experimentation is routine. See *PPG Indus., Inc. v. Guardian Indus. Corp.*, 75 F.3d 1558, 1564, 37 USPQ2d 1618, 1623 (Fed. Cir. 1996); *In re Wands*, 858 F.2d 731, 736-37, 8 USPQ2d 1400, 1403-07 (Fed. Cir. 1988) (applying this principle in the context of monoclonal antibody production); and MPEP §§ 2164.01, 2164.06. As for the concern regarding working examples, it is accepted that an application need not describe what is already known in the art. *See, e.g., Lindemann Machinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 221 USPQ 481 (Fed. Cir. 1984).

Methods for generating antibodies to a specifically provided polypeptide sequence, and in accordance with the present claims, are well know in the art.

Accordingly, the Applicant submits that the pending claims fall within the subject matter that is enabled and described by the specification. In particular, a specific CIP/CIP fragment comprising a PTH antagonist is identified. Accordingly, the Applicant respectfully requests reconsideration and withdrawal of this rejection.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 7-9 and 19-25 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention.

The claims are amended herein to identify a specific CIP/CIP fragment and to remove the language objected to by the Office. Accordingly, the Applicant respectfully requests reconsideration and withdrawal of the present rejection.

Restriction Status

As requested by the Office, the Applicant indicates that, as used in the present application, and specifically limited thereto, the terms "CIP" and "CIP fragment" are the same and are not patentably distinct.

Supplemental Declaration

A supplemental Declaration/Power of Attorney in accordance with 37 C.F.R. § 1.63 is submitted concurrently with the present amendment. This Declaration/Power of Attorney includes a priority claim to the earlier filed U.S. provisional application number 60/224,447, filed August 10, 2000.

CONCLUSION

The Applicant believes that all issues raised in the Office Action have been properly addressed in this response. Accordingly, reconsideration and allowance of the pending claims is respectfully requested. If the Examiner feels that a telephone interview would serve to facilitate resolution of any outstanding issues, the Examiner is encouraged to contact Applicant's representative at the telephone number below.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, Applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 532212001500.

Dated: May 24, 2004

Respectfully submitted,

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Activation-independent Parathyroid Hormone Receptor Internalization Is Regulated by NHERF1 (EBP50)*

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Parathyroid hormone (PTH) regulates extracellular calcium homeostasis through the type 1 PTH receptor (PTH1R) expressed in kidney and bone. The PTH1R undergoes β-arrestin/dynamin-mediated endocytosis in response to the biologically active forms of PTH, PTH-(1–34), and PTH-(1–84). We now show that amino-truncated forms of PTH that do not activate the PTH1R nonetheless induce PTH1R internalization in a cell-specific pattern. Activation-independent PTH1R endocytosis proceeds through a distinct arrestin-independent mechanism that is operative in cells lacking the adaptor protein Na/H exchange regulatory factor 1 (NHERF1) (ezrin-binding protein 50). Using a combination of radioligand binding experiments and quantitative, live cell confocal microscopy of fluorescently tagged PTH1Rs, we show that in kidney distal tubule cells and rat osteosarcoma cells, which lack NHERF1, the synthetic antagonist PTH-(7–34) and naturally circulating PTH-(7–84) induce internalization of PTH1R in a β-arrestin-independent but dynamin-dependent manner. Expression of NHERF1 in these cells inhibited antagonist-induced endocytosis. Conversely, expression of dominant-negative forms of NHERF1 conferred internalization sensitivity to PTH-(7–34) in cells expressing NHERF1. Mutation of the PTH1R PDZ-binding motif abrogated interaction of the receptor with NHERF1. These mutated receptors were fully functional but were now internalized in response to PTH-(7–34) even in NHERF1-expressing cells. Removing the NHERF1 ERM domain or inhibiting actin polymerization allowed otherwise inactive ligands to internalize the PTH1R. These results demonstrate that NHERF1 acts as a molecular switch that legislates the conditional efficacy of PTH fragments. Distinct endocytic pathways are determined by NHERF1 that are operative for the PTH1R in kidney and bone cells.

Extracellular calcium homeostasis in vertebrate animals is primarily under the endocrine control of the parathyroid hormone (PTH)[1]/type I PTH receptor (PTH1R). The PTH1R, predominantly expressed in kidney and bone cells, belongs to class B of the large superfamily of G protein-coupled receptors (GPCRs) that consists of receptors for peptide hormones and neuropeptides (1). Class B GPCRs are characterized by a common topology and by their ability to couple to multiple signaling pathways via distinct G proteins.

PTH is synthesized by the parathyroid glands as a mature peptide of 84 amino acids that is stored in secretory vesicles and dense core granules. Reductions of extracellular calcium levels are detected by the calcium-sensing receptor on parathyroid chief cells and promote the release of PTH, which acts on bone (to increase resorption) and kidney (to augment reabsorption), thereby restoring serum calcium levels. PTH-(1–84) is usually the major form of PTH secreted by the parathyroid glands. However, recent analyses reveal that PTH fragments that are likely to be PTH-(7–84) are also secreted by the parathyroid glands and generated by peripheral metabolism (2, 3). These PTH fragments or their synthetic analogs are thought to be inactive on the PTH1R because, despite binding to the receptor, they fail to promote activation of the classical effectors adenylyl cyclase and phospholipase C (4–7). In fact, NH_2-terminally truncated PTH fragments behave as competitive PTH antagonists (8).

As with most GPCRs, the responses of the PTH1R to agonists are regulated by multiple mechanisms, including a well characterized and highly conserved process involving receptor phosphorylation by G protein-coupled receptor kinase 2 (9, 10) and arrestin recruitment (11–13). These processes contribute directly to PTH1R desensitization by facilitating the uncoupling of the receptor from its cognate G proteins, G_s and G_q. Following desensitization, the PTH1R is endocytosed into intracellular compartments, from which it can be either recycled to the membrane, leading to receptor resensitization

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‖ Recipient of a CIHR doctoral award.

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[1] The abbreviations used are: PTH, parathyroid hormone; PTH1R, type 1 PTH and PTH-related peptide receptor; hPTH1R, human PTH1R; GPCR, G protein-coupled receptor; EBP50, ezrin-binding protein 50; NHERF1, Na/H exchange regulatory factor 1; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; DCT, distal convoluted tubule; PCT, proximal convoluted tubule; SaOS, human osteosarcoma cells; ROS, rat osteosarcoma cells; HA, hemagglutinin.

(14), or targeted for degradation, leading to receptor down-regulation (15, 16).

Increasing evidence demonstrates that PTH1R activation and endocytosis can be dissociated, with each event requiring distinct and specific receptor conformational states. PTH peptide analogs that efficiently activate the PTH1R but fail to induce arrestin-mediated internalization have been described (17). Conversely, PTH1R mutants have been generated that exhibit impaired ability to transduce G protein-mediated signaling but are phosphorylated by G protein-coupled receptor kinase 2 and internalized in response to PTH-(1–34) (18). These observations raise the possibility that PTH analogs that are unable to activate the PTH1R may be capable of inducing receptor endocytosis. In the present work, we tested this hypothesis. We now show that amino-terminally truncated PTH peptides internalize the PTH1R without antecedent or concomitant activation. We uncovered the molecular mechanism underlying this novel phenomenon and found that it occurs in a cell-specific manner that depends on the expression of the scaffolding protein EBP50, also known as NHERF1.[2]

NHERF1 is a cytoplasmic adaptor protein that contains tandem PDZ domains that have been implicated in protein targeting and in the assembly of protein complexes (19, 20). NHERF1 also possesses an ERM domain, which binds to the actin-associated proteins, ezrin, radixin, moesin, and merlin (21). NHERF1 recruits various cellular receptors, ion transporters, and other proteins to the plasma membrane of epithelia and other cells (22). Recently, Mahon and co-workers (23) reported that NHERF1 and -2 bind to the PTH1R through a COOH-terminal PDZ recognition motif and determined a role for NHERF2 in PTH signaling. Our findings demonstrate a novel action for NHERF1 as a molecular switch that determines the conditional efficacy of PTH fragments in bone and kidney cells. Additionally, the results establish the existence of distinct endocytic pathways for the PTH1R in response to either agonists or nonactivating PTH fragments. As such, they provide a new cellular mechanism for the regulation of GPCR function.

EXPERIMENTAL PROCEDURES

Cell Culture—The preparation, subcloning, characterization, and culture conditions for kidney and bone cells were as described (24, 25). Kidney cells were grown in a 50:50 mix of Dulbecco's modified Eagle's medium/Hamm's F-12 (10-092-CV; Mediatech, Inc., Herndon, VA), supplemented with 5% heat-inactivated fetal calf serum (Invitrogen) and 1% PSN (5 mg of penicillin, 5 mg of streptomycin, and 10 mg of neomycin/ml; Invitrogen). SaOS2 cells were grown in RPMI supplemented with 10% fetal calf serum. HEK-293 and ROS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transient transfections of HEK-293 cells grown to 75–90% confluence were performed using FuGENE 6™ (Roche Applied Science) according to the manufacturer's instructions. Empty pcDNA3 vector was added to keep constant the total DNA amount added per plate. Cells grown on 100-mm (DCT) and 60-mm (ROS) plates were transfected using 6 μg of total DNA. DCT and ROS cells stably expressing NHERF1 were generated by transiently transfecting NHERF1 cDNA in pcDNA 3.1 Hygro using FuGENE 6™. After 48 h, transiently transfected cells were trypsinized and plated on 150-mm dishes containing culture medium supplemented with 300 μg/ml hygromycin (Invitrogen) to select stable transfectants.

Complementary DNA Constructs—pEGFP-N2 plasmid encoding a full-length human PTH1R carboxyl-terminal EGFP fusion protein (PTH1R/C-EGFP) was kindly provided by C. Silve (INSERM, Paris, France). The PTH1R with EGFP introduced in the E2 extracellular domain (PTH1R/N-EGFP) has been previously described (26).

Mutation of the terminal amino acid of PTH1R, in PTH1R/C-EGFP, from methionine to alanine (M593A) was performed by PCR using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). The fidelity of the construct was confirmed by sequencing (ABI PRISM 377; Applied Biosystems, Foster City, CA) and subsequent sequence alignment (NCBI BLAST) with human PTH1R (GenBank™ accession number XM 002895).

Cyclic AMP and Inositol Phosphate Assays—Ligand-stimulated accumulation of cAMP was determined in the presence of 1 μM 3-isobutyl-1-methylxanthine. Phosphatidylinositol hydrolysis was determined in the presence of 10 mM LiCl. cAMP and total inositol phosphates were measured chromatographically as previously described (17).

Receptor Internalization—PTH1R internalization was measured by quantitative, live cell confocal fluorescence microscopy in cells stably transfected with the hPTH1R-EGFP. Cells were plated on poly-D-lysine-coated 25-mm glass coverslips and analyzed at room temperature by confocal microscopy (Amersham Biosciences) equipped with a 488-nm argon/krypton laser. Emitted fluorescence was detected with a 515–540-nm band pass filter. PTH1R/EGFP internalization was analyzed by selecting the entire plasma membrane through a plane normal to and ~2–3 μm above the basal membrane surface (ImageScan; Amersham Biosciences). Sequential images were acquired at 1-min intervals. After obtaining three control images, the indicated ligand was introduced, and images were obtained for an additional 15–30 min to ensure that internalization was complete with any given maneuver. Internalization of PTH1R/EGFP was reflected by a loss of plasma membrane fluorescence, quantified as changes in pixel intensity. Fluorescence intensity was digitized at 16-bit resolution and converted to 256 grayscale levels for each image. The product of the number of pixels within the defined membrane area and the average pixel intensity was calculated for each time point. Kinetic parameters were determined by fitting the data to a sigmoidal nonlinear equation, where PTH1R internalization = bottom + (top − bottom)/(1 + $10^{(\log EC_{50} - \log[PTH])}$) and plotted using Prism (GraphPad Software, Inc.). Results are presented as the mean ± S.E. for the indicated number of independent observations.

Radioligand Binding and Internalization—Cells (100,000–200,000) prepared as described above were incubated on ice for 2 h with ~100,000 cpm of high pressure liquid chromatography-purified [^{125}I][Nle^{8,18},Tyr^{34}]PTH-(1–34)NH$_2$ in 250 μl of Dulbecco's modified Eagle's medium/F-12 medium containing 5% fetal bovine serum, essentially as described (27, 28). In brief, cells grown to confluence in 24-well plates were incubated for 2 h at room temperature (to achieve equilibrium binding). Under these conditions, the concentration of radioligand was ~0.1 nM. Following incubation, the cells were washed twice with ice-cold phosphate-buffered saline and collected in 0.5 ml of 0.1 N NaOH, and bound [^{125}I]PTH was assessed by γ spectrometry. Ligand internalization was measured as follows. Cells (100,000–200,000) were washed twice with ice-cold phosphate-buffered saline and incubated in 0.5 ml of Dulbecco's modified Eagle's medium/F-12 medium containing 5% fetal bovine serum at room temperature. At the indicated time points, surface-bound ligand was extracted by two 5-min incubations on ice with 50 mM glycine buffer (pH 3.0) containing 0.1 M NaCl. After acid extraction, the remaining cell-associated (internalized) radioligand was collected in 0.5 ml of 0.1 N NaOH. The amount of radioligand in each fraction was assessed by γ spectrometry. Radioligand internalization is expressed as the ratio (percentage) of internalized fraction over the total cell-associated ligand (surface plus internalized). Nonspecific binding and internalization were measured in parallel experiments carried out in the presence of 1 μM unlabeled PTH-(1–34). Curves were fit using a four-point logistic algorithm (Prism, GraphPad Software, San Diego, CA).

Arrestin Translocation—DCT cells grown on 100-mm dishes were transiently transfected with 1 μg of β-arrestin-2/GFP and 5 μg of hPTH1R-pcDNA3 (courtesy of Dr. Marc Caron, Duke University). After 48 h, the cells were split onto collagen-coated 25-mm coverslips. Arrestin translocation in response to PTH ligands was assessed at room temperature using real time live cell confocal microscopy as reported (29).

Dynamin Dependence—DCT cells were split onto 25-mm coverslips and transiently transfected with 1 μg of PTH1R/EGFP in the presence of 1 μg of K44A-dynamin-pcDNA3.1 (Dr. Orson Moe, University of Texas, Dallas) or empty pcDNA3.1 vector. PTH1R internalization in response to PTH ligands was then measured and quantified as outlined previously.

Coimmunoprecipitation—Six-well plates of HEK-293 cells were transfected with the different combinations of DNA constructs as indicated. Forty-eight h after transfection, the cells were rinsed with ice-cold phosphate-buffered saline and harvested in 800 μl of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM $Na_2P_2O_7$, 5 mM EDTA) supplemented with protease inhibitors (9 nM pepstatin, 9 nM antipain, 10 nM leupeptin, and 10 nM

[2] In the present work, we use the terms NHERF1 (EBP50) and NHERF2 (E3KARP) to distinguish between the two forms of NHERF.

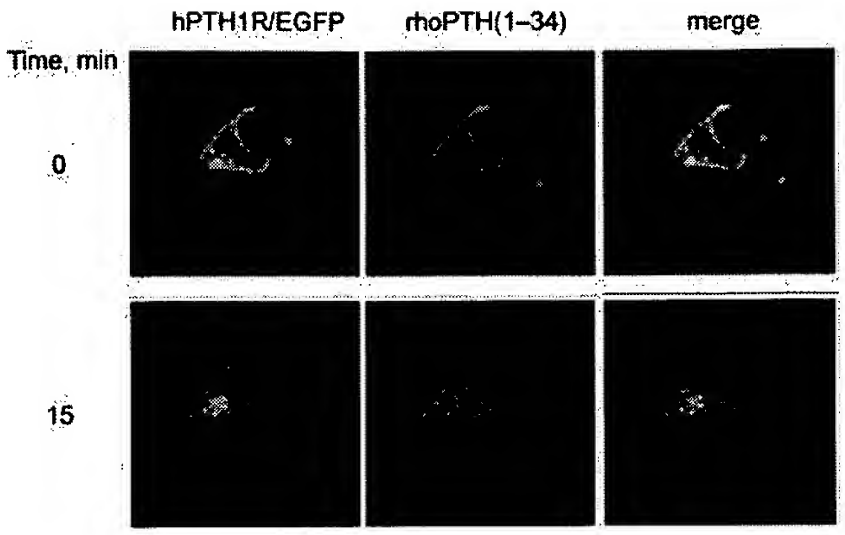


FIG. 1. **Colocalization of hPTH1R/EGFP and PTH-(1–34).** A single DCT cell stably expressing hPTH1R/EGFP is shown immediately upon (0 min) and 15 min after the addition of 10^{-7} M Rho-PTH-(1–34). At time 0, hPTH1R/EGFP (*green*) was largely membrane-delimited, although some trans-Golgi and perinuclear localization is visible. Rho-PTH-(1–34) (*red*) bound to PTH1R/EGFP on the cell membrane. Merge of hPTH1R/EGFP and Rho-PTH-(1–34) depicts regions where the receptor and ligand colocalize (*yellow*). After 15 min of treatment with Rho-PTH-(1–34), plasma membrane fluorescence was reduced, whereas fluorescence of both the PTH1R/EGFP and Rho-PTH-(1–34) increased and colocalized within the cytoplasm.

chymostatin) (Sigma). After lysis for 60 min at 4 °C, the lysates were clarified by centrifugation for 20 min at 14,000 rpm at 4 °C. Four μg of specific anti-GFP polyclonal antibody (Molecular Probes, Inc., Eugene, OR) were added to the supernatant. After a 60-min incubation at 4 °C, 50 μl of 50% protein A-agarose pre-equilibrated in lysis buffer was added, followed by an overnight incubation at 4 °C. Samples were then centrifuged for 1 min in a microcentrifuge and washed three times with 1 ml of lysis buffer. Immunoprecipitated proteins were eluted by the addition of 50 μl of SDS sample buffer followed by a 30-min incubation at room temperature. Initial lysates and immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting using specific antibodies.

Immunoblot Analysis—Cells were grown to confluence in T-25 flasks, trypsinized, and collected by centrifugation. The resultant cell pellet was resuspended in 500 μl of Nonidet P-40 lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). Total protein concentrations were measured (Bio-Rad Dc Protein Assay). 30 μg of lysate (solubilized in Laemmli sample buffer) were resolved on 10% polyacrylamide gels by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore Corp.) according to standard methods. Membranes were blocked overnight at 4 °C with 5% nonfat dried milk in Tris-buffered saline plus Tween-20 (TBST), incubated with polyclonal anti-EBP50 antibody (Affinity Bioreagents) at 1:1000 dilution for 4 h at room temperature, washed, and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce) at 1:5000 dilution for 1 h at room temperature. Protein bands were visualized with a luminol-based enhanced chemiluminescence substrate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistics—Data are presented as means ± S.E., where n indicates the number of independent experiments. Effects of experimental treatments were assessed by paired comparisons within experiments and reported as the mean ± S.E. of n independent experiments. Paired results were compared by analysis of variance with post-test repeated measures analyzed by the Bonferroni procedure. Single comparisons to control were analyzed by Dunnett's test (Prism; GraphPad). Differences greater than $p \leq 0.05$ were assumed to be significant.

RESULTS

PTH1R and PTH Endocytosis—The PTH1R and PTH-(1–34) were simultaneously localized in DCT cells stably expressing a human PTH1R COOH-terminally tagged with the enhanced green fluorescent protein (PTH1R/C-EGFP) as previously described (17, 28, 30). Cells were exposed to rhodamine-tagged PTH-(1–34) (*rhoPTH(1–34)* in Fig. 1). Initially, the PTH1R was largely limited to the plasma membrane, although some cytoplasmic and perinuclear fluorescence is evident (Fig. 1). The ligand was restricted to the plasma membrane. When the fluorescence images for receptor (*green*) and ligand (*red*) were merged, only PTH1R at the plasma membrane was associated with ligand (*yellow*). After 15 min, little PTH1R or PTH-(1–34) remained at the plasma membrane. The decreases of PTH1R and PTH fluorescence at the plasma membrane were accompanied by concomitant increases of cytoplasmic PTH1R/C-EGFP and rhodamine-labeled PTH-(1–34) fluorescence. These results are consistent with the view that PTH and the PTH1R colocalize and internalize together in response to receptor occupancy.

Cell- and Ligand-specific Internalization of the PTH1R—We determined the kinetics of PTH1R internalization by real time, quantitative confocal fluorescence microscopy monitoring of membrane-delimited fluorescence intensity of the PTH1R/C-EGFP fusion protein in live cells. Upon the addition of 10^{-7} M PTH-(1–34), PTH1R internalization began after a latency of 6–7 min and reached 50% at 15 min (Fig. 2*A*). The results obtained on single cells by fluorescent imaging were independently corroborated by measuring internalization of radiolabeled PTH-(1–34) in large (>100,000) populations of cells. Ligand internalization paralleled that of the receptor. During the first 5 min, less than 5% of [^{125}I]PTH-(1–34) was sequestered and 50% was endocytosed by 15 min (Fig. 2*B*). These findings demonstrate that the PTH1R and PTH-(1–34) traffic together and are internalized in a spatially and temporally congruent manner. The results qualitatively and quantitatively validate the optical determination of PTH1R endocytosis.

PTH-(1–34) also internalized the PTH1R in kidney proximal tubule (PCT) cells (Fig. 2*A*). Internalization began promptly without delay in PCT cells but by 15 min reached levels equivalent (50%) to that in DCT cells.

We next examined the effects of the PTH antagonist PTH-(7–34) on PTH1R internalization. As expected, PTH-(7–34) (10^{-7} M) did not promote PTH1R internalization in PCT cells (Fig. 2*C*). However, PTH-(7–34) promptly and efficiently induced receptor sequestration in kidney DCT cells. PTH1R endocytosis evoked by PTH-(7–34) was greater (82 ± 4.2 *versus* 49 ± 3.7%, $p < 0.01$) and more rapid (t = 2.5 *versus* >8 min) than that elicited by PTH-(1–34). From concentration dependence curves, half-maximal internalization (EC_{50}) of the PTH1R occurred at 0.90×10^{-9} M PTH-(1–34) and 10^{-8} M for PTH-(7–34). We confirmed that PTH-(7–34) lacks agonist activity (31, 32). In DCT cells, 10^{-7} M PTH-(7–34) had no effect on either adenylyl cyclase or phospholipase C, whereas PTH-(1–34) activated both adenylyl cyclase and phospholipase C (21.4 ± 0.1- and 3.2 ± 0.5-fold, respectively). These results additionally substantiate that EGFP ligation to the intracellular tail of the PTH1R does not interfere with its signaling in DCT cells, as in HEK-293 cells or COS cells (17).

To verify that the observed ligand-specific internalization effects of PTH-(7–34) were not due to the presence of EGFP on the intracellular tail of the PTH1R/C-EGFP, identical experiments were performed with a PTH1R, where EGFP is located in the extracellular domain (PTH1R/N-EGFP) (26). We previously established that this receptor construct signals and traffics normally in response to PTH-(1–34) in LLC-PK_1 kidney cells (26). PTH1R/N-EGFP internalization induced by PTH-(7–34) was 76 ± 8.7% at 15 min and did not differ from that of the PTH1R/C-EGFP, 81 ± 4.2%. PTH-(1–34) also efficiently internalized the PTH1R/N-EGFP. Thus, the functional properties of the PTH1R/C-EGFP and PTH1R/N-EGFP are not different from the native receptor.

The actions of PTH-(1–84) and PTH-(7–84) were assessed to determine if the full-length circulating forms of PTH exerted similar effects on PTH1R internalization. At comparable peptide concentrations (10^{-7} M), these naturally occurring forms of PTH evoked PTH1R endocytosis in DCT cells that was indis-

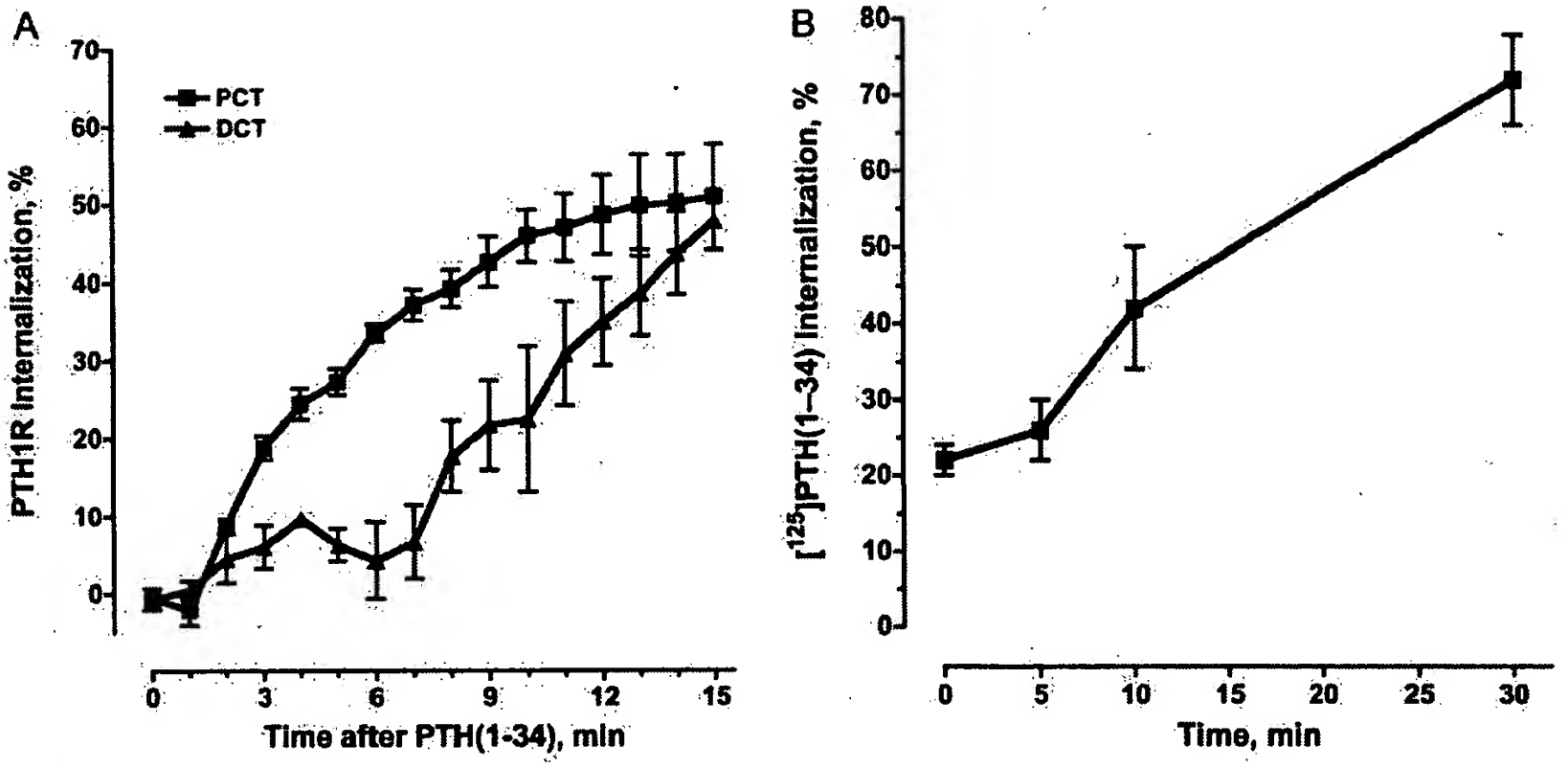


FIG. 2. **Ligand-induced PTH1R endocytosis.** *A*, effect of 10^{-7} M PTH-(1–34) on PTH1R internalization in PCT and DCT convoluted tubule cells. Receptor endocytosis was measured by real time quantitative confocal microscopy. Confocal images were quantified at 60-s intervals as described under "Experimental Procedures." $n = 3$ for PCT; $n = 4$ for DCT. *B*, radioligand internalization of PTH-(1–34) in DCT cells. Results are the means ± S.D. of triplicate determinations in two independent experiments. *C*, cell-specific PTH1R internalization by PTH-(7–34) in DCT but not PCT cells. 10^{-7} M PTH-(7–34) was added at 0 min. $n = 3$ for DCT; $n = 3$ for PCT. *D*, effect of full-length PTH peptides (10^{-7} M) on PTH1R endocytosis in DCT cells. *E*, ligand-induced PTH1R endocytosis in bone cells. PTH-(7–34) evokes PTH1R endocytosis in ROS cells but not in SaOS2 cells. Receptor endocytosis was measured after the addition of 10^{-7} M PTH-(1–34) or PTH-(7–34) as described. The extent of receptor endocytosis after 15 min is depicted. $n = 3$ for ROS; $n = 3$ for SaOS2.

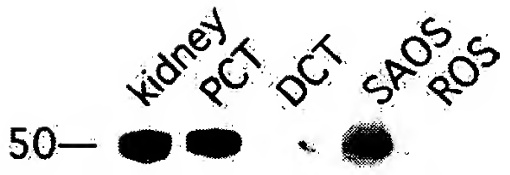


FIG. 3. **NHERF1 expression.** Immunoblot of mouse kidney, PCT, DCT, human osteosarcoma SaOS2, and rat osteosarcoma ROS 17/2.8 cells. Lanes were loaded with 5 μg (kidney) or 20 μg (cell lines) of protein per lane.

tinguishable from that displayed by their respective shorter synthetic analogs (Fig. 2*D*).

Similar cell-specific effects of PTH-(1–34) and PTH-(7–34) on PTH1R internalization were observed in two bone-derived cell lines, human SaOS2 and rat ROS 17/2.8 cells (Fig. 2*E*). In SaOS2 cells, as in PCT kidney cells, only PTH-(1–34) induced PTH1R internalization. In contrast, in ROS cells both PTH-(1–34) and PTH-(7–34) induced PTH1R endocytosis, similar to kidney DCT cells.

NHERF1 Expression Determines PTH-(7–34) Effects on PTH1R Endocytosis—NHERF1 is expressed by PCT and SaOS2 cells (Fig. 3), where PTH-(7–34) has no effect on PTH1R internalization, but not by DCT or ROS cells (Fig. 3), where PTH-(7–34) induces endocytosis (33, 34). Therefore, we theorized that the presence or absence of NHERF1 determines the cell-specific pattern of internalization of PTH1R in response to inactive PTH peptides.

To test this idea, we introduced NHERF1 in cells normally lacking it and determined the effect of PTH-(7–34) on PTH1R internalization. DCT and ROS cells were stably transfected with NHERF1. DCT/NHERF1 cells exhibited PTH1R internalization in response to PTH-(1–34) similar to that seen in vector-transfected or non-transfected control cells (Fig. 4*A*). Now, however, PTH1R internalization in response to PTH-(7–34) was significantly attenuated (Fig. 4*A*). PTH-(7–34)-induced PTH1R internalization was also largely inhibited in ROS cells stably expressing NHERF1 (Fig. 4*B*). PTH-(1–34) and PTH-(7–34) promoted the internalization of a PTH1R tagged with EGFP in the extracellular domain (PTH1R/N-EGFP) (Fig. 4*C*). NHERF1 similarly inhibited PTH-(7–34)-induced internalization of the PTH1R/N-EGFP as it did the PTH1R/C-EGFP (Fig. 4*C*). PTH-(1–34)-stimulated PTH1R/N-EGFP endocytosis was not affected. Thus, the actions of NHERF1 are independent of the location of EGFP within the PTH1R.

The role of NHERF1 in determining sensitivity to PTH-(7–34) was further and independently established by expressing a dominant negative form of NHERF1 (NHERH-(1–326), NHERF1ΔERM) (35, 36) in PCT cells that endogenously express NHERF1. Cells transfected with NHERF1ΔERM now displayed PTH1R internalization in response to PTH-(7–34) (Fig. 5), whereas they are normally refractory to PTH-(7–34), as shown by the cells transfected with empty vector.

Direct Interaction between NHERF1 and PTH1R Determines PTH-(7–34) Effects on PTH1R Endocytosis—The interaction between PTH1R and NHERF1 was directly demonstrated in HEK-293 cells co-expressing EGFP-tagged wild-type PTH1R with HA-tagged NHERF1. HEK-293 cells were used because they are normally devoid of PTH1R and have been previously employed for GPCR coimmunoprecipitation with NHERF1 (37, 38). Cell lysates were immunoprecipitated with a GFP-specific polyclonal antibody, and blotting was performed with an HA-specific monoclonal antibody. As shown in Fig. 6 (*top*), NHERF1 efficiently coimmunoprecipitated with the PTH1R. This finding also establishes that such interaction occurs constitutively (*i.e.* without ligand occupancy).

We then generated a full-length PTH1R/EGFP construct wherein the terminal methionine of the PTH1R was changed to

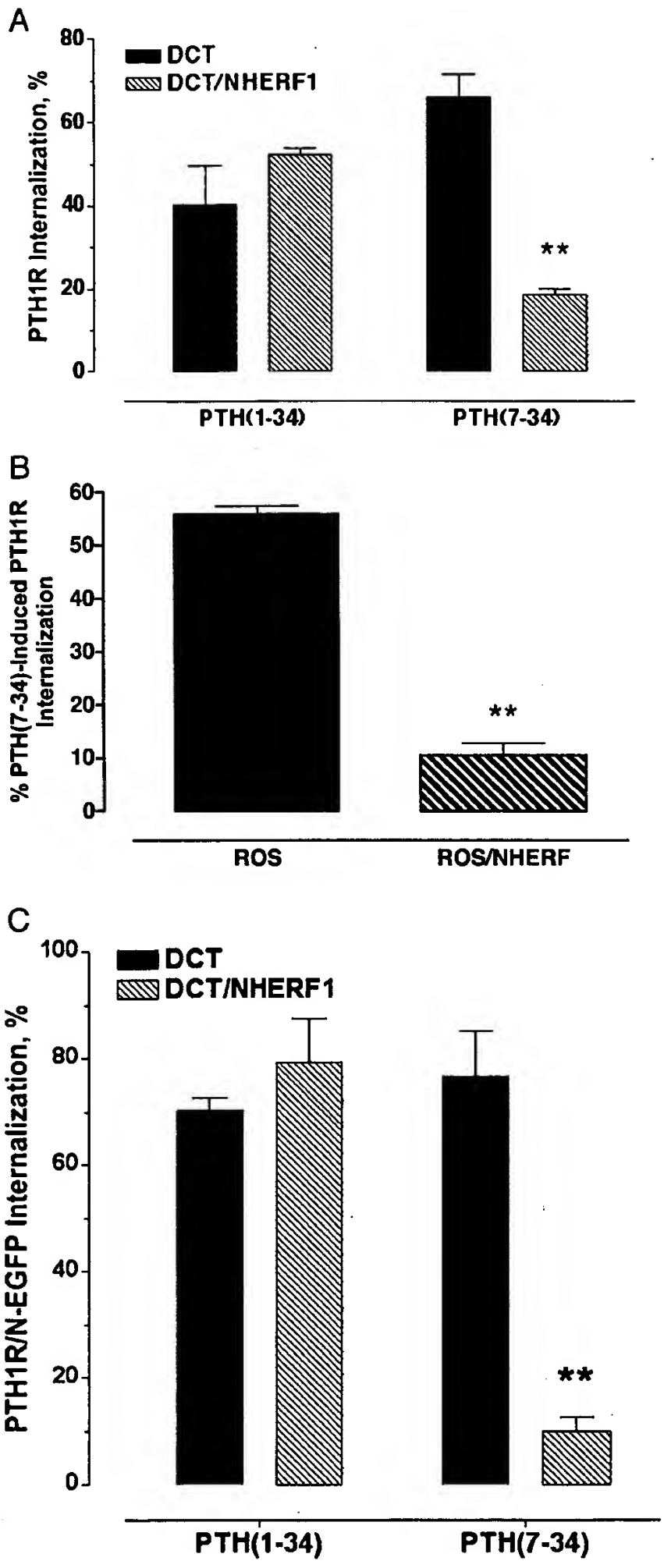


FIG. 4. **NHERF expression inhibits PTH1R endocytosis in DCT and ROS cells.** *A*, effect of 10^{-7} M PTH-(1–34) or PTH-(7–34) on PTH1R internalization in DCT cells after 15 min in the absence (*filled bars*) or presence (*stippled bars*) of NHERF1 ($n = 3$). **, $p < 0.01$ *versus* DCT. *B*, effect of 10^{-7} M PTH-(7–34) on PTH1R internalization in ROS cells in the absence (*filled bars*) or presence (*stippled bars*) of NHERF1. Receptor endocytosis was measured and quantified as outlined in Fig. 1*A*. The results show the average ± S.E. of three independent observations for each condition. **, $p < 0.01$ *versus* ROS. *C*, PTH-(7–34)-induced internalization of the PTH1R with an extracellular EGFP tag (PTH1R/N-EGFP). $n = 3$. **, $p < 0.01$ *versus* PTH-(1–34).

alanine (M593A) (ETVA-PTH1R/C-EGFP), a modification that disrupts the interaction of the PTH1R with the PDZ domain of NHERF1 (23). The ETVA-PTH1R/C-EGFP was expressed in a functional form and stimulated cAMP production in response to 10^{-7} M PTH-(1–34) similarly to wild type PTH1R (7.5 ± 0.5- and 8.5 ± 0.7-fold above basal level for ETVA-PTH1R and wild-type PTH1R, respectively).

In contrast to the wild-type ETVM-PTH1R, however, ETVA-PTH1R coimmunoprecipitation with NHERF1 was negligible (Fig. 6, *top*). Immunoblotting of the cell lysates with an anti-GFP antibody showed that both wild-type and ETVA-PTH1R were expressed at similar levels (Fig. 6, *middle*). Likewise, immunoblotting of the cell lysates with the HA-specific antibody showed that HA-NHERF1 expression levels were similar in all conditions (Fig. 6, *bottom*). Thus, mutation of a single residue of the PDZ recognition domain was sufficient to disrupt the association of the PTH1R with NHERF1. The results further demonstrate that the presence of EGFP at the carboxyl terminus of the PTH1R does not occlude or interfere with the PDZ-recognition domain, binding to NHERF1, or PTH1R signaling.

The ETVA-PTH1R was used to test the hypothesis that disrupting the PDZ recognition domain of the PTH1R, which prevents binding to NHERF1, would permit internalization in response to PTH-(7–34). This was accomplished by introducing the ETVA-PTH1R/C-EGFP in PCT cells that constitutively express NHERF1. Fig. 7*A* shows that 10^{-7} M PTH-(7–34), which normally has a negligible effect on PTH1R internalization, now effectively internalized the ETVA-PTH1R. PTH-(1–34) stimulated internalization of the ETVA-PTH1R as efficiently as that of the wild-type PTH1R. Comparable results were obtained with ROS/NHERF cells (Fig. 7*B*). These experiments provide strong evidence that the PDZ-binding domain of the PTH1R is necessary and sufficient to mediate the association with NHERF1. Moreover, they establish that disrupting this interaction confers sensitivity to PTH-(7–34).

Role of ERM Domain on PTH1R Endocytosis—NHERF1 possesses a carboxyl-terminal ERM domain. To determine whether the ERM domain of NHERF1 is required for ligand-induced PTH1R internalization, we expressed ERM-deficient NHERF1 (NHERFΔERM) in DCT cells. In contrast to full-length NHERF1, which substantially inhibited PTH1R internalization initiated by PTH-(7–34), NHERFΔERM exerted no significant inhibitory action (Fig. 8). This finding is consistent with the idea that NHERF1 tethers the PTH1R to the actin cytoskeleton through the ERM domain of NHERF1 and, in the absence of the ERM domain, the PTH1R is unconstrained. In this setting, receptor occupancy by PTH is sufficient to induce endocytosis.

The ERM domain of NHERF1 binds actin-associated proteins (39). We reasoned that if the PTH1R is tethered through NHERF to ezrin and the actin cytoskeleton, disrupting the actin cytoskeleton should unleash the PTH1R from NHERF1 and permit PTH-(7–34) to internalize the receptor. We tested this theory in PCT cells because they express NHERF1 and because PTH-(7–34) normally has a minimal effect on PTH1R endocytosis. Treatment with 1 μM cytochalasin D, a membrane-permeant inhibitor of actin polymerization, fully allowed PTH-(7–34) to promote receptor internalization (Fig. 9). Since the ETVA-PTH1R does not interact with NHERF1, cytochalasin D should not interfere with PTH-(7–34)-initiated PTH1R sequestration. As predicted, actin depolymerization did not alter PTH-(7–34)-stimulated internalization of the ETVA-PTH1R (Fig. 9). In contrast to the inhibitory action of cytochalasin D on PTH (7–34)-induced PTH1R internalization, microtubule disruption with colchicine (1 μM) had no effect on PTH1R inter-

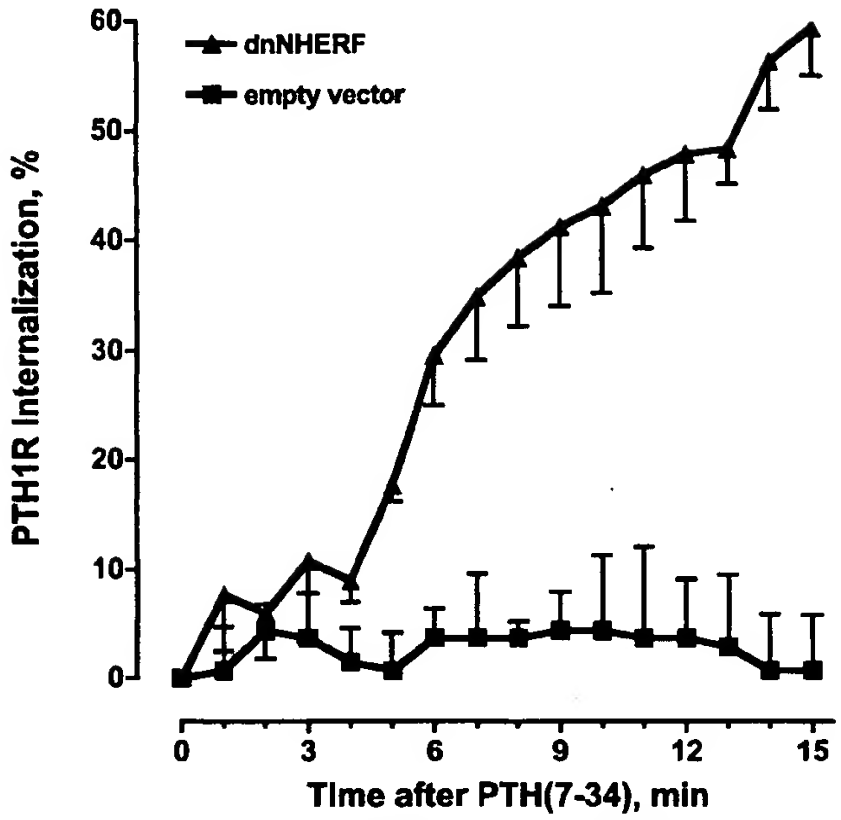


FIG. 5. **ERM-deficient dominant negative NHERF (NHERF-(1–326)) permits PTH-(7–34) to internalize the PTH1R in PCT cells.** The effect of 10^{-7} M PTH-(7–34) on PTH1R internalization in PCT cells in the presence or absence of NHERF-(1–326). Receptor endocytosis was measured and quantified as outlined in Fig. 1*A*. $n = 4$ for each condition.

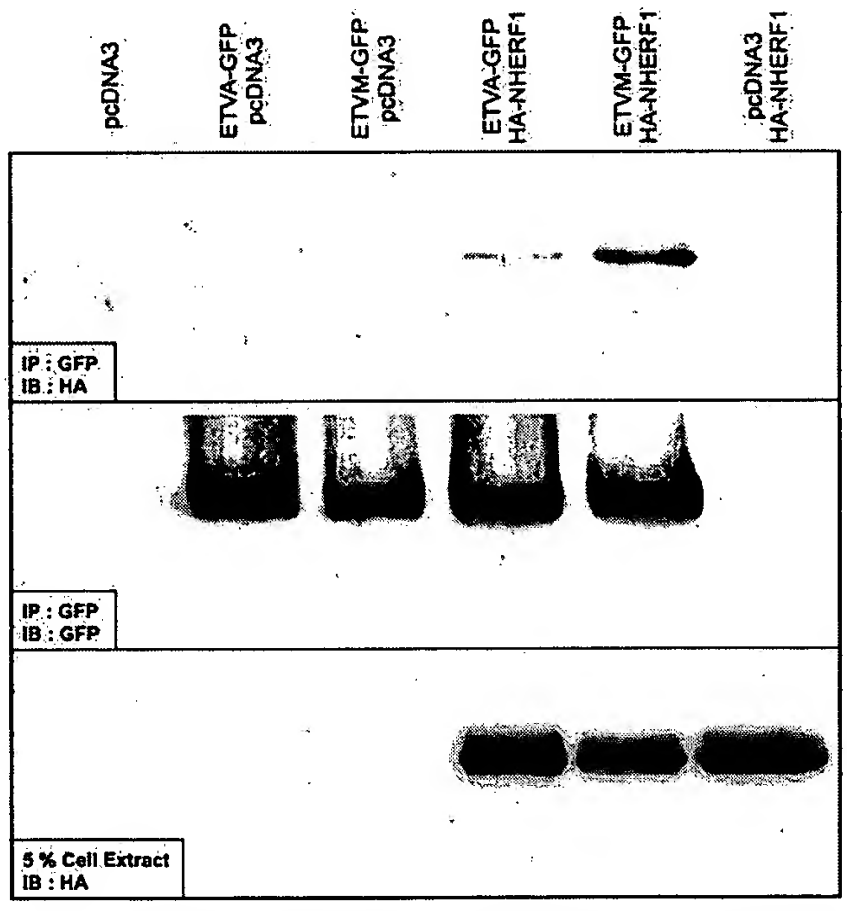
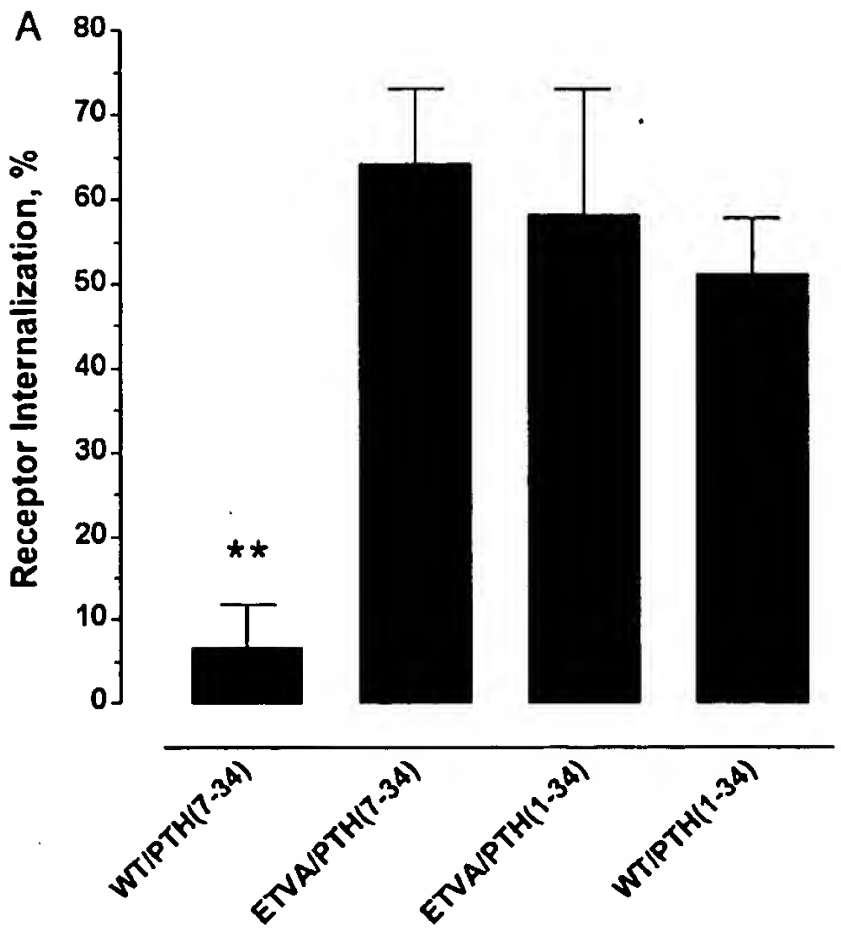


FIG. 6. **Coimmunoprecipitation of EBP50 with PTH1R(ETVM)-EGFP and PTH1R(ETVA)-EGFP.** HEK-293 cells transfected with the indicated constructs were harvested, lysed, and immunoprecipitated as described under "Experimental Procedures." Immunoprecipitation experiments were performed by incubating the cell lysates with an EGFP-specific polyclonal antibody followed by incubation with protein A-agarose. Immunoprecipitated proteins were eluted from protein A-agarose with SDS sample buffer. Eluates and cell extracts were subjected to SDS-PAGE. Immunoblotting was performed with a HA-specific monoclonal antibody (*upper panel*). The amount of immunoprecipitated EGFP-tagged receptor in each sample was verified by immunoblotting with the EGFP-specific polyclonal antibody (*middle panel*). The quantity of HA-EBP50 present in each cell extract was evaluated by immunoblotting with the HA-specific monoclonal antibody (*lower panel*). A representative Western blot is shown.



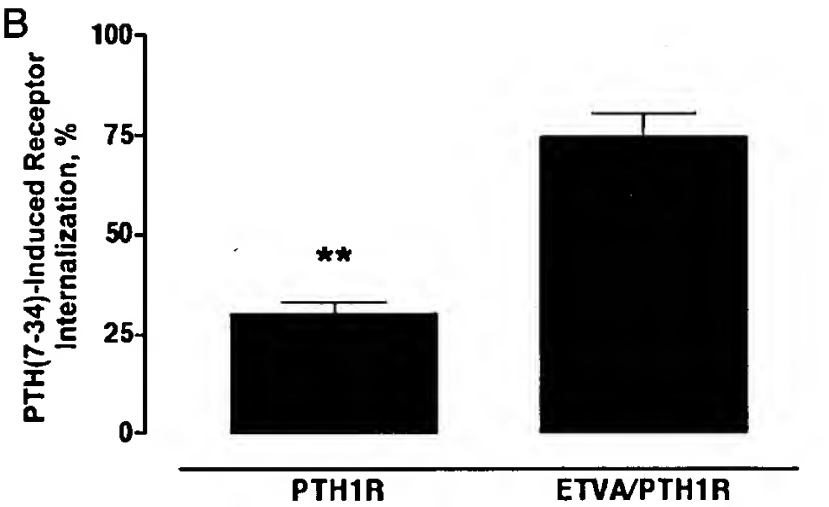


FIG. 7. **Mutation of the carboxyl-terminal PDZ interaction domain in the PTH1R abolishes NHERF-mediated inhibition of PTH-(7–34)-induced PTH1R endocytosis.** *A*, PCT cells expressing either the wild-type (*WT*) PTH1R/EGFP or mutated (*ETVA*) PTH1R/EGFP were challenged with 10^{-7} M PTH-(1–34) or PTH-(7–34) for 15 min as indicated. $n = 3$–4 for each condition. **, $p < 0.01$ *versus* wild-type PTH-(1–34). *B*, ROS/NHERF cells expressing either the wild-type PTH1R/EGFP ($n = 4$) or mutated ETVA-PTH1R/EGFP ($n = 3$) were challenged with 10^{-7} M PTH-(7–34), and endocytosis was measured as described. **, $p < 0.01$ *versus* ETVA-PTH1R.

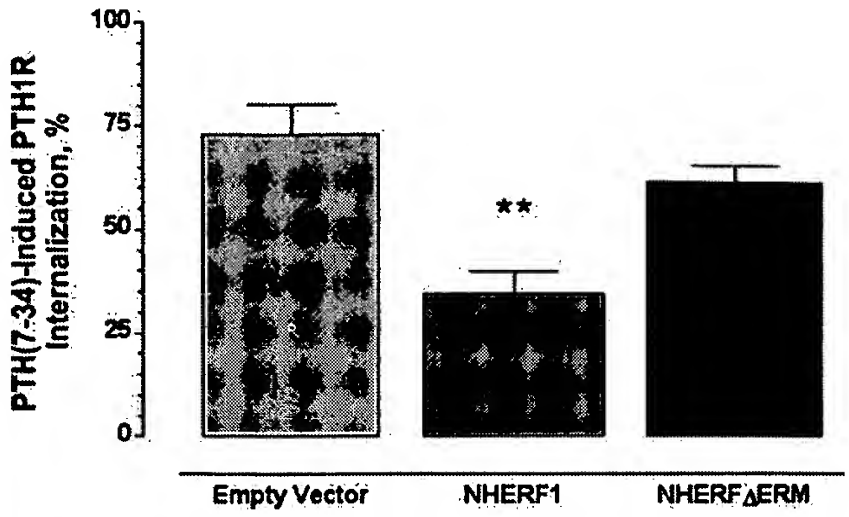


FIG. 8. **ERM-deficient NHERF-(1–326) does not inhibit PTH-(7–34)-induced PTH1R endocytosis in DCT cells.** DCT cells were transiently transfected with PTH1R/EGFP in the presence of pcDNA 3.1 (empty vector), NHERF1, or ERM-deficient NHERF-(1–326), and PTH1R internalization was assessed after challenge with 10^{-7} M PTH-(7–34) for 15 min. $n = 3$ for each condition. **, $p < 0.01$ *versus* empty vector.

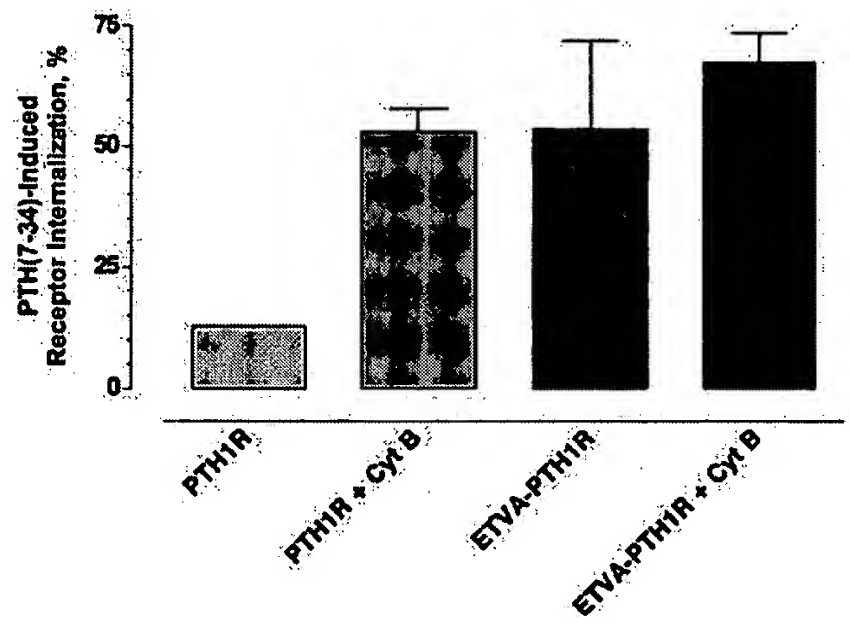


FIG. 9. **Disruption of the actin cytoskeleton permits PTH-(7–34)-induced PTH1R internalization in PCT cells.** PCT cells were transiently transfected with PTH1R/EGFP or ETVA-PTH1R/EGFP. Where indicated, cells were treated with 1 μM cytochalasin D for 15 min before the addition of 10^{-7} M PTH-(7–34). Receptor endocytosis was then measured after 15 min and quantified as described. Results show internalization at $t = 15$ min.

nalization (data not shown). These results support the view that the presence of an intact actin network and NHERF1 determine the fate of PTH1R trafficking in response to activating or inactivating PTH peptides.

PTH-(7–34)-induced PTH1R Internalization Is Independent of β-Arrestin but Requires Dynamin—To define further the molecular mechanisms underlying antagonist-induced PTH1R endocytosis in DCT cells, we sought to determine whether PTH1R sequestration involves β-arrestin and dynamin. DCT cells were transiently transfected with β-arrestin-2-GFP and monitored by fluorescence microscopy. Under resting conditions, β-arrestin-2 was uniformly distributed throughout the cytoplasm but excluded from the nucleus (Fig. 10, *top*). Within 5 min of the addition of 10^{-7} M PTH-(1–34), β-arrestin-2 moved from the cytoplasm to the plasma membrane, exhibiting a characteristic punctate distribution. By 25 min, β-arrestin had translocated to the cytoplasm. This observation is consistent with arrestin-mediated trafficking of the PTH1R to clathrin-coated pits for internalization. In contrast, PTH-(7–34) (10^{-7} M) exerted no detectable effect on β-arrestin-2 movement (Fig. 10, *bottom*).

DCT cells expressing PTH1R-EGFP were transfected with a dominant-negative form of dynamin, [K44A]dynamin (40). In these cells, PTH1R internalization was significantly inhibited, both in response to the agonist PTH-(1–34) and to the antagonist PTH-(7–34) (Fig. 11). Hence, whereas agonist-occupied PTH1R internalizes in a classical β-arrestin- and dynamin-dependent fashion, receptor occupancy by the nonactivating analog PTH-(7–34) induces PTH1R endocytosis independently of β-arrestin. Dynamin function, however, is required.

DISCUSSION

A feature common to GPCRs is the cyclical process of activation, desensitization and internalization, resensitization, and recycling to the plasma membrane (41). These coordinated events protect against excessive receptor stimulation or periods of prolonged inactivity. In this manner, receptor activation,

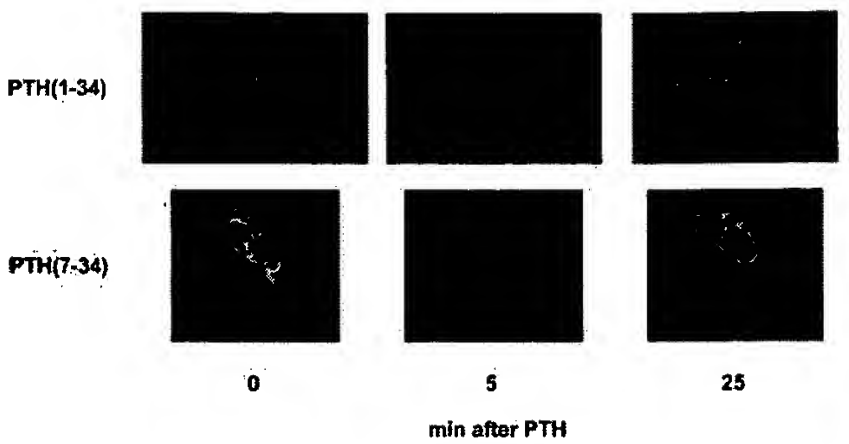


FIG. 10. **PTH-(1–34) translocates β-arrestin-2 in DCT cells.** DCT cells were transiently transfected with β-arrestin-2-GFP and hPTH1R as outlined under "Experimental Procedures." Real time, live cell confocal images were taken after 0, 5, and 25 min of treatment with 10^{-7} M PTH-(1–34) or -(7–34), as indicated.

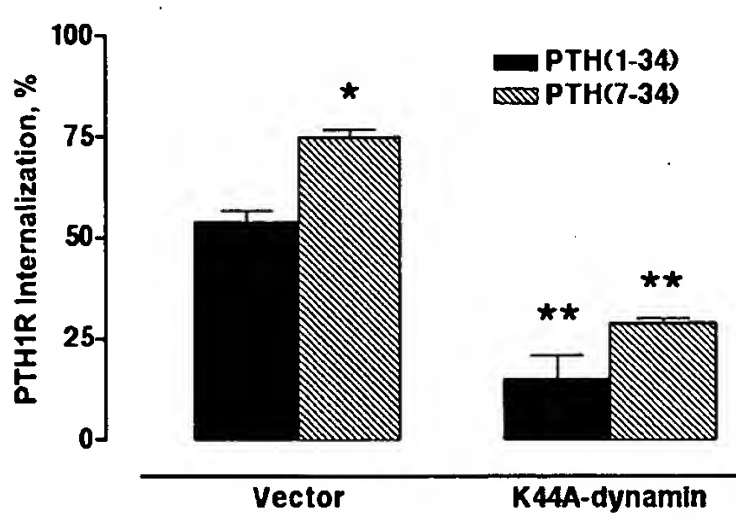


FIG. 11. **PTH1R endocytosis is dynamin-dependent.** DCT cells were transiently transfected with PTH1R/EGFP in the presence of dominant negative K44A-dynamin or empty vector. PTH1R internalization was analyzed after treatment for 15 min with 10^{-7} M PTH-(1–34) or PTH-(7–34) as indicated. $n = 3$ independent observations for each condition. *, $p < 0.05$; **, $p < 0.01$ *versus* vector plus PTH-(1–34).

desensitization, and trafficking are thought to go hand-in-hand, thereby regulating the physiological balance of GPCR activity. In some instances, however, GPCR activation can be dissociated from subsequent desensitization and internalization. For the PTH1R, this has been shown with synthetic analogs of the PTH-related protein that stabilized an active, G protein-coupled PTH1R state. In this configuration, persistent signaling is maintained, and the receptor is not competent to interact with β-arrestin-2 and is not desensitized or internalized (17). Conversely, PTH1R mutants have been generated that exhibit severely blunted signaling activities but are phosphorylated by G protein-coupled receptor kinase 2 and endocytosed in a β-arrestin-2-dependent manner (18). Similar findings were reported following mutagenesis of β_2-adrenergic (42) and angiotensin type 1 receptors (43).

This raises the important question of whether ligands that bind but do not activate signaling through G proteins are capable of inducing receptor sequestration. In the present studies, this possibility was explored by using structural analogs of PTH on cells derived from kidney (proximal and distal tubule cells) and bone (SaOS2 and ROS cells), since these organs are major targets of PTH action. We found that PTH1R internalization occurs in response to NH_2-terminally truncated fragments of PTH that do not activate signaling either through G_s or G_q proteins. Strikingly, this PTH1R internalization occurs in a markedly cell-specific fashion and proceeds through a mechanism that is distinct from that induced by PTH agonists. The agonist PTH-(1–34) activated the PTH1R, mobilized β-arrestin, and internalized the receptor, as previously shown in HEK-293, COS-7, and LLC-PK_1 cells (11, 44, 45). PTH-(7–34), a competitive inhibitor of the PTH1R, however, robustly promoted receptor internalization in DCT and ROS cells without accompanying activation or β-arrestin-2 translocation. These results demonstrate that PTH1R activation and internalization can be dissociated in a ligand- and cell-selective fashion. Some precedent for this phenomenon may be found with the class A serotonin, endothelin, and cholecystokinin GPCRs, where both agonists and synthetic antagonists internalize these receptors (46–48). It has been suggested that the distinct biological effects of different ligands acting through a common GPCR partially depend upon their abilities to induce endocytosis (49). Thus, the dissociation between receptor activation and internalization as shown here may represent a more common biological phenomenon that contributes to ligand- and cell-specific hormone and drug action for multiple classes of GPCRs.

NHERF1 has been shown previously to affect the function of some GPCRs containing PDZ-binding motifs. NHERF1, for instance, enhances the rate of recycling of K-opioid (36) and β_2-adrenergic receptors (50). Disrupting the interaction of NHERF1 with the β_2-adrenergic receptor causes sorting of endocytosed receptor to the lysosomal pathway instead of the recycling pathway (50). Segre and co-workers (23) reported that NHERF1 and -2 bind to the PTH1R through a COOH-terminal PDZ-binding domain (ETVM) and determined a role for NHERF2 in PTH signaling. The present work illustrates a different role of NHERF1 that is distinct from its effect on receptor recycling, on the one hand, and apparently unrelated to the signaling switch, on the other.

The capacity of NHERF1 to establish cell-specific effects on PTH1R internalization was tested in three independent ways. In the first, we mutated the PDZ-binding domain of PTH1R by changing the terminal methionine to alanine (M593A). This mutation is sufficient to abolish the association of the full-length PTH1R with NHERF1 (Fig. 6). The ETVA-PTH1R was fully functional, and, as expected, in cells lacking NHERF1 was endocytosed equivalently to the wild-type PTH1R by both PTH-(1–34) and PTH-(7–34). The ETVA-PTH1R, however, was also efficiently internalized in response to PTH-(7–34) in cells expressing NHERF1, whereas the wild-type PTH1R was not. Thus, interfering with the ability of the PTH1R to associate with NHERF1 is sufficient to permit the nonsignaling PTH-(7–34) to internalize the receptor.

PTH1R fusion proteins containing EGFP within the extracellular domain or at the carboxyl-terminus of the receptor were used. These receptor constructs exhibit signaling behavior that is indistinguishable from the native receptor (26, 30). As shown here, the EGFP-tagged receptors were both internalized in response to PTH-(7–34), and this effect was absent in cells constitutively expressing or transfected with NHERF1. Further, the wild-type PTH1R sequence COOH-terminally ligated to EGFP efficiently coimmunoprecipitated with NHERF1. Single residue mutagenesis of the terminal Met of the PTH1R was sufficient to abrogate interaction of the PTH1R/C-EGFP with NHERF1. Therefore, the interaction of NHERF1 with the PTH1R/EGFP fusion protein involves the ETVM recognition motif within the PTH1R sequence. These findings further indicate that COOH-terminal ligation of EGFP does not interfere with the physical interaction or functional effects of NHERF1 with the PTH1R. In this regard, the EGFP-tagged PTH1R behaves like certain other proteins such as nNOS that recognize internal PDZ motif-mediated interactions (51). An 18-amino acid linker between the COOH terminus of the PTH1R sequence and the start of the EGFP sequence may

facilitate interaction between the PDZ recognition motif and NHERF1. The two PDZ domains of NHERF1 can dimerize, preferentially through homologous binding interactions (52, 53). NHERF1 dimerization may permit interaction with non-canonical COOH-terminal PDZ recognition motifs as in the PTH1R/EGFP fusion protein.

The second strategy to examine the role of NHERF1 in regulating PTH1R endocytosis involved using a truncated form of NHERF1 that lacks the ERM domain (NHERF-(1–326)) but contains both PDZ domains. In cells expressing NHERF1, NHERF-(1–326) exerted a dominant-negative function and permitted PTH-(7–34) to internalize the PTH1R. Furthermore, whereas introduction of full-length NHERF1 in cells normally lacking it suppressed the effect of PTH-(7–34) (Fig. 4, *A* and *B*), expression of NHERF-(1–326) alone had no effect (Fig. 8). These results further establish a role for the NHERF1 ERM domain in tethering the PTH1R and indicate that interfering with the association between PTH1R, NHERF1, and the actin cytoskeleton allows the occupied, but not activated, receptor to be endocytosed.

ERM proteins such as NHERF1 contain an F-actin binding site in the COOH-terminal 28 residues (39). Ezrin, a member of the ERM family, cross-links the actin cytoskeleton to the plasma membrane. Ezrin is abundantly expressed at the apical brush-border membrane of proximal tubules, the site of NHERF1 localization (34, 54, 55). Ezrin is likewise expressed by osteosarcoma cells (56). Therefore, the third tactic that was applied to test the role of NHERF1 in determining the effects of inactive ligands on PTH1R internalization was to disrupt the actin cytoskeleton. Application of cytochalasin D to cells possessing NHERF1 allowed PTH-(7–34) to internalize the PTH1R. This effect was quite specific for the actin cytoskeleton, since microtubule disruption with colchicine had no effect on PTH1R internalization. These independent approaches provide strong evidence that NHERF1 dictates the response of the PTH1R to occupancy by nonactivating PTH peptides. In cells lacking NHERF1, PTH1R occupancy is sufficient to promote receptor internalization without prior or concurrent activation.

The mechanism of PTH1R internalization in response to PTH-(7–34) in cells lacking NHERF1 is, at least in part, different from that commonly employed by the same receptor in response to agonists. Agonist-induced endocytosis of the PTH1R occurred in an arrestin-dependent manner. In the case of nonactivating analogs, PTH1R sequestration proceeds in a β-arrestin-independent manner. In both instances, however, internalization requires dynamin. This distinct internalization pathway is operative in cells lacking NHERF1 or in cells expressing NHERF1ΔERM. Taken together, these findings suggest that the role of NHERF1 is not necessarily an "active" one but rather that the interactions between the PTH1R, NHERF1, and cytoskeleton (through the ERM domain) confer sufficient membrane stability on the PTH1R to require full agonist occupancy for internalization. This possibility is supported by the observation that the interaction between PTH1R and NHERF1 is constitutive, since both proteins coimmunoprecipitated from preparations of nontreated cells.

It is now apparent that PTH1R activation, desensitization, and internalization can be dissociated with each event requiring distinct receptor conformational states. These specific states can be selectively stabilized by appropriate modifications of the ligand. PTH analogs containing specific modifications at the NH_2 terminus have been shown to efficiently activate the PTH1R, but they fail to induce arrestin-mediated internalization (17). The postactivation response of the PTH1R depends on specific interactions between the NH_2-terminal activation domain of the ligand and the third extracellular loop of the receptor (17). These interactions are distinct from those necessary for activation of G protein-mediated signal transduction. The present work shows that the presence of the adaptor protein NHERF1 and its interaction with the PTH1R legislates the cell-specific pattern of PTH1R internalization in response to otherwise inactive PTH fragments. Taken together, these observations indicate that PTH1R activation and desensitization/endocytosis are mediated through distinct structural states that derive from specific interactions between ligand and receptor. Thus, agonist- or antagonist-occupied receptor states induce distinct conformations or accessibility to intracellular domains. The differential or inducible involvement of these domains in coupling to G proteins may represent a molecular basis for ligand-selective responses not only for the PTH1R but also for other GPCRs. In the case of the PTH1R, these conformational states also depend on interactions between the PTH1R and NHERF1 at the cytoplasmic surface.

In addition to its relevance to GPCR regulation and trafficking, the present studies may have implications for understanding extracellular calcium homeostasis. After synthesis of mature PTH-(1–84), the protein is concentrated in secretory vesicles and granules. Morphologically distinct granule subtypes contain both PTH and the proteases cathepsin B and cathepsin H (59). The co-localization of proteases and PTH in secretory granules may explain the observation that a portion of the PTH secreted from parathyroid glands consists of amino-terminally truncated PTH fragments (60). These fragments do not activate the PTH1R. Therefore, the intracellular fragmentation of PTH is thought to represent an inactivating pathway to dispose of "excess" peptide in situations such as hypercalcemia (61, 62). As shown here (Fig. 2*D*), PTH-(1–84) and PTH-(7–84) exerted actions on PTH1R internalization comparable with their shorter respective synthetic analogs. This novel finding suggests that PTH-(7–84) is not so much an inactive peptide as an inactivating protein.

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Human PTH-(7-84) Inhibits Bone Resorption *in Vitro* Via Actions Independent of the Type 1 PTH/PTHrP Receptor

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The linear sequence of intact mammalian PTH consists of 84 amino acids, of which only the most amino(N)-terminal portion, *i.e.* PTH-(1-34), is required for the classical actions of the hormone on mineral ion homeostasis mediated by the type 1 PTH/PTHrP receptor (PTH1R). Like the N-terminus, the carboxyl (C)-terminal sequence of PTH is highly conserved among species, and various circulating PTH C-fragments are generated by peripheral metabolism of intact PTH or are directly secreted, in a calcium-dependent manner, by the parathyroid glands. Certain synthetic PTH C-fragments exert actions on bone and cartilage cells that are not shared by PTH-(1-34), and specific binding of PTH C-peptides has been demonstrated in bone cells in which PTH1R expression was eliminated by gene targeting. The peptide human (h) PTH-(7-84) recently was shown to inhibit the calcemic actions of hPTH-(1-34) or hPTH-(1-84) in parathyroidectomized animals. To determine whether this anticalcemic effect of hPTH-(7-84) *in vivo* might result from direct actions on bone, we studied its effects on both resorption of intact bone *in vitro* and formation of osteoclasts in primary cultures of murine bone marrow. Human (h) PTH-(7-84) (300 nM) reduced basal 72-h release of preincorporated ^{45}Ca from neonatal mouse calvariae by 50% (9.6 ± 1.9% *vs.* 17.8 ± 5.7%; $P < 0.001$) and similarly inhibited resorption induced by hPTH-(1-84), hPTH-(1-34), 1,25-dihydroxyvitamin D_3 (VitD), PGE_2, or IL-11. In 12-d murine marrow cultures, both hPTH-(7-84) (300 nM) and hPTH-(39-84) (3000 nM) lowered VitD-dependent formation of osteoclast-like cells by 70%. On the contrary, these actions of hPTH-(7-84) were not observed with the PTH1R antagonists hPTH-(3-34)NH_2 and [L^{11},D-W^{12},W^{23},Y^{36}]hPTHrP-(7-36)NH_2, which, unlike hPTH-(7-84), did inhibit PTH1R-dependent cAMP accumulation in ROS 17/2.8 cells. We conclude that hPTH-(7-84), acting via receptors distinct from the PTH1R and presumably specific for PTH C-fragments, exerts a direct antiresorptive effect on bone that may be partly due to impaired osteoclast differentiation. (*Endocrinology* 143: 171–176, 2002)

INTACT PTH from different mammalian species comprises 84 amino acids, the sequence of which is highly conserved within both its amino (N)-terminal and carboxyl (C)-terminal regions (1). The first 34 amino acids of PTH, including an intact N-terminus, are both necessary and sufficient for the classical actions of the hormone on mineral ion homeostasis and bone metabolism. These effects of intact and N-terminal PTH are mediated through the type 1 PTH/PTH-related peptide receptor (PTH1R), a G protein-coupled receptor that can activate both adenylate cyclase and PLC (2).

The parathyroid glands are the main source of PTH, although small amounts of its mRNA were recently identified in hypothalamus and spleen (3). PTH synthesis and secretion are tightly controlled by calcium via a membrane-bound calcium-sensing receptor (4), although vitamin D (5, 6) and phosphate (7, 8) also play modulating roles. Under physiological conditions, a portion of the newly synthesized hormone undergoes intraglandular cleavage at a rate that also is regulated by extracellular calcium (9, 10). This cleavage results in the cosecretion of intact PTH and various C-terminal fragments, the predominant forms of which, identified to date, consist of peptides with N-termini located between residues 24 and 43 (11–13). Secreted intact PTH also undergoes endopeptidic cleavage(s) in peripheral tissues, mainly liver and kidney, by processes that degrade the resulting N-terminal fragments *in situ* but release additional C-fragments into the circulation (14–16). As a consequence of their obligatory renal clearance, the concentration of circulating C-terminal PTH (CPTH) fragments increases dramatically in patients with renal failure (17–19).

Recently, fragments of PTH lacking residues at the extreme N-terminus but otherwise large enough to cross-react with most commercially available intact PTH two-site immunoassays were detected after HPLC fractionation of normal plasma and, at much higher levels, in plasma of patients with advanced renal failure (19). Although their precise structure(s) has not been ascertained, these fragments exhibit chromatographic properties similar to those of synthetic PTH-(7-84) (18). Interestingly, human (h) PTH-(7-84) was recently shown to inhibit the calcemic actions of PTH-(1-84) and PTH-(1-34) in parathyroidectomized animals at doses much lower than would be predicted to effectively antagonize either hormonal form at the PTH1R (20, 21). Thus, these *in vivo* observations suggest that CPTH fragments might act upon bone cells via one or more mechanisms independent of the PTH1R *per se*.

The possibility that CPTH fragments (as well as intact PTH) might activate receptors distinct from the PTH1R was first postulated over 2 decades ago when Arber *et al.* (22) showed that a particular CPTH fragment, PTH-(53-84), possessed biological properties different from those of PTH-(1–34). Subsequent work from several different groups has produced direct evidence that CPTH fragments from within the sequence PTH-(35-84) bind specifically to bone and kidney cells and/or membranes and can exert direct actions on target cells in bone or cartilage. For example, CPTH frag-

Abbreviations: CPTH, C-terminal PTH; hPTH, human PTH; PTH1R, type 1 PTH/PTHrP receptor; TRAP, tartrate-resistant acid phosphatase; TRAP+MNC, TRAP-positive cells containing three or more nuclei (osteoclast-like multinucleated cells); VitD, 1,25-dihydroxyvitamin D_3.

ments such as hPTH-(53-84) and hPTH-(60-84) increased alkaline phosphatase activity and expression of mRNAs for both alkaline phosphatase and osteocalcin in bone-derived cells and induced transient increases in cytosolic free calcium in chondrocytes (23–26). Photoaffinity cross-linking studies to characterize the receptors for CPTH fragments (*i.e.* CPTHRs) expressed by ROS 17/2.8 osteosarcoma and rPT parathyroid cells were performed by Inomata *et al.* (27) using radioiodinated ($Leu^{8,18}$,Tyr^{34})hPTH-(1-84) and (Tyr^{34})hPTH-(19-84), neither of which binds well, if at all, to the PTH1R. These studies showed that in ROS 17/2.8 cells, two proteins (80 and 30 kDa) interacted specifically with the radioligands used, whereas in rPT cells, only the 80-kDa protein was observed. Affinity labeling was inhibited by hPTH-(1-84), hPTH-(19-84), and, to a lesser extent, by CPTH fragments that were truncated even further at the N-terminus, whereas hPTH-(1-34) had no effect (27). Recently, hPTH-(7-84) was shown to bind to CPTHRs on ROS 17/2.8 cells with affinity comparable to that of hPTH-(1-84) (21).

Unequivocal evidence that such CPTHRs are distinct from the PTH1R was provided by our recent demonstration that specific [^{125}I](Tyr^{34})hPTH-(19-84) binding is observed in clonal osteoblastic and osteocytic cell lines derived from mice in which the PTH1R gene had been eliminated by gene targeting (28). Further, CPTH fragments such as hPTH-(39-84) were shown to regulate cellular functions (*i.e.* connexin 43 expression and apoptosis) in clonal PTH1R-null osteocytes at concentrations shown to bind effectively to CPTHRs in these cells (28).

Thus, the expression of CPTHRs in bone offers a plausible mechanism by which circulating PTH fragments, truncated at their N-termini and including peptides as long as hPTH-(7-84) might exert biological actions, potentially different from those of intact PTH, by a means other than direct antagonism at the PTH1R. To determine whether the ability of hPTH-(7-84) to antagonize the calcemic response to PTH-(1-84) *in vivo* might reflect direct actions of this C-PTH fragment on bone, we studied its effects using *in vitro* assays of osteoclast formation and bone resorption.

Materials and Methods

Materials

Culture media were obtained from the Media Kitchen (Pediatric Surgery, Massachusetts General Hospital, Boston, MA), other tissue culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY), and additional reagents and chemicals were obtained from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA). Recombinant hPTH-(1-84) was a gift from Chugai Pharmaceutical Co. (Shizuoka, Japan), and hPTH-(7-84) and [D^{76}]hPTH-(39-84) were purchased from Bachem (Torrance, CA). All other PTH fragments, including the PTH1R antagonist (Leu^{11},D-Trp^{12},Trp^{23},Tyr^{36})hPTHrP-(7-36)amide PTHrP-(7-36) (29, 30) and hPTH-(3-34)-amide, were synthesized at Massachusetts General Hospital Peptide and Oligonucleotide Core Laboratory (Boston, MA). Recombinant mouse IL-11 was purchased from R&D Systems, Inc. (Minneapolis, MN), and VitD was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

Animals

Animals were maintained in facilities operated by the Massachusetts General Hospital Center for Comparative Resources in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were employed using protocols approved by the institutional animal care and use committee.

Bone resorption assay

Bone resorption was quantitated by the release of previously incorporated ^{45}Ca from newborn mouse calvarial bones *in vitro* (31). Briefly, calvaria from 3- to 4-d-old mice (CD-1 strain, Charles River Laboratories, Inc., Wilmington, MA) were obtained after maternal administration of 50 μCi $^{45}CaCl_2$ (NEN Life Science Products, Boston, MA), sc, on the 19th day of gestation. The bones were divided in half and precultured in 1 ml DMEM containing 1 mM calcium, 2 mM phosphate, 5% heat-inactivated horse serum, and 1% antibiotic/antimycotic solution (Life Technologies, Inc.) on a rocking platform at 90 oscillations/min in a 37 C incubator under 5% CO_2 in air. After 24 h the medium was replaced with 1 ml fresh medium containing the test substances (or vehicle alone). After an additional 72 h, the bones were removed, rinsed three times in PBS, placed in scintillation vials containing 0.4 ml 2 N HCl, and incubated for 2 h at room temperature before addition of 5 ml scintillation fluid (Packard Instruments, Downers Grove, IL). Aliquots of culture medium (0.5 ml) were transferred to separate vials containing 5 ml scintillation fluid for determination of released radioactivity. In some experiments additional aliquots of culture medium were used for measurements of cAMP as described below. Bone resorption was determined as the percentage of total initial bone ^{45}Ca subsequently released into the medium during the 72-h treatment period. Results are expressed as the mean ± SEM of the percentage of ^{45}Ca released for groups of four bones and are representative of at least three independent experiments.

Bone marrow culture

Bone marrow cells were isolated as previously described (32). Briefly, 4- to 6-wk-old male mice (C57B/6 strain, Charles River Laboratories, Inc.) were killed by carbon dioxide asphyxiation, and tibias and femurs were aseptically removed and dissected free of adhering tissue. The metaphyses were removed, and the marrow cavity was flushed with 1 ml αMEM to obtain marrow cells, which were collected into 50-ml tubes and washed twice with αMEM. Cells were cultured in growth medium [αMEM containing 10% FBS (lot 1011961 Life Technologies, Inc.) and 1% penicillin-streptomycin] containing 100 nM dexamethasone (Sigma) after plating at 1.5×10^6 cells/well in 24-well plates. Half of the culture medium was replaced 3 times/wk with fresh medium containing a 2× concentration of the test substances (or vehicle). All cultures were maintained in a 37 C incubator under 5% CO_2 in air. After culture for 10 d, cells adherent to the surface of each well were rinsed twice with PBS, fixed with 10% formalin in PBS for 10 min at room temperature and with ethanol/acetone (50:50, vol/vol) for 1 min before staining for tartrate-resistant acid phosphatase (TRAP), as previously described (33). TRAP-positive cells containing 3 or more nuclei were scored as osteoclast-like multinucleated cells (TRAP+MNCs). Cells were counted at ×10 magnification in 20 contiguous fields along 2 orthogonal pathways in each well, a method previously employed to account for the nonuniform distribution of cells within wells (33). The number of TRAP+MNCs contained in these 20 fields was expressed as the number per well.

cAMP accumulation

Clonal rat osteosarcoma cell (ROS 17/2.8) were cultured in 48-well plates in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin. The cultures were maintained for 5–7 d after reaching confluence by replacing the medium every other day. To assess basal and agonist-induced cAMP accumulation, cells were rinsed twice with assay buffer (DMEM containing 2 mM isobutylmethylxanthine, 1 mg/ml heat-inactivated BSA, and 35 mM HEPES-NaOH, pH 7.4) and then incubated for 45 min at 23 C with the same buffer alone or in the presence of different peptides (or with conditioned medium collected from resorption assays). The buffer then was rapidly aspirated, the plates were frozen on powdered dry ice, and the frozen cells were subsequently thawed directly into 0.25 ml 50 mM HCl. Cell-associated cAMP in the acid extracts was measured as previously described (34). Results were expressed as picomoles of cAMP produced per well over 45 min.

Statistical analysis

Results are expressed as the mean ± SEM or the mean ± SD. The significance of differences between treatment and control groups was

assessed by the Mann-Whitney test. Data were analyzed using the PRISM 3.0 software package for Macintosh (GraphPad Software, Inc., San Diego, CA).

Results

The limited efficacy of short, amino-terminally truncated PTH or PTHrP analogs, such as hPTH-(3-34) or hPTHrP-(7-36), to antagonize the action of PTH-(7-34) or PTH-(1-84) *in vivo* or *in vitro* contrasts with the efficiency with which hPTH-(7-84) inhibits the calcemic action of hPTH-(1-84) [or hPTH-(7-34)] *in vivo* (20, 21). Although pharmacokinetic differences *in vivo* between N-truncated PTH fragments of different length and structure might explain these differences, we considered the alternative possibility that, unlike short N-truncated PTH or PTHrP fragments, this effect of hPTH-(7-84) *in vivo* might not be mediated via antagonism at the PTH1R. We therefore directly compared the effects of hPTH-(7-84) with those of PTHrP-(7-36) or hPTH-(3-34) in an *in vitro* assay of bone resorption that relies upon the release of pre-incorporated ^{45}Ca from neonatal murine calvarial bones. First, as shown in Fig. 1, addition of hPTH-(7-84) (300 nM) alone reduced basal ^{45}Ca release by approximately 50% [control, 17.8 ± 5.7%; hPTH-(7-84), 9.6 ± 1.9%; $P < 0.001$]. This effect was comparable to that of salmon calcitonin (100 nM; 9.9 ± 1.1%; $P < 0.001$). In contrast, no inhibition of basal resorption was observed with equimolar concentrations of much shorter N-truncated PTH analogs, such as hPTH-(3–34) (300 nM; 18.7 ± 4.2%) or PTHrP-(7-36) (300 nM; 15.4 ± 4.9%), that bind as well or more effectively to the PTH1R as hPTH-(7-84).

Further, as shown in Fig. 2, hPTH-(7-84) (300 nM) also significantly inhibited (by 50% or more) agonist-induced bone resorption caused by a variety of osteotropic agents, including intact hPTH-(1-84) (3 nM), hPTH-(1-34) (3 nM), VitD (10 nM), PGE_2 (100 nM), and IL-11 (10 ng/ml). The antiresorptive effect of hPTH-(7-84) was dose dependent, with an IC_{50} of approximately 200 nM (data not shown). We considered the possibility that these inhibitory actions of hPTH-(7-84) might reflect antagonism, at the PTH1R, of an effect of locally secreted PTHrP to augment the responses to these other agonists. As shown in Fig. 3, however, neither hPTH-(3-34) (1 μM) nor PTHrP-(7-36) (1 μM), both of which act as PTH1R antagonists (see below) at the concentrations used, inhibited resorption induced by VitD (10 nM). With respect to a possible nonspecific or irreversible toxic effect of hPTH-(7-84), we observed that removal of the peptide after 24 h of exposure to calvariae did not impair the

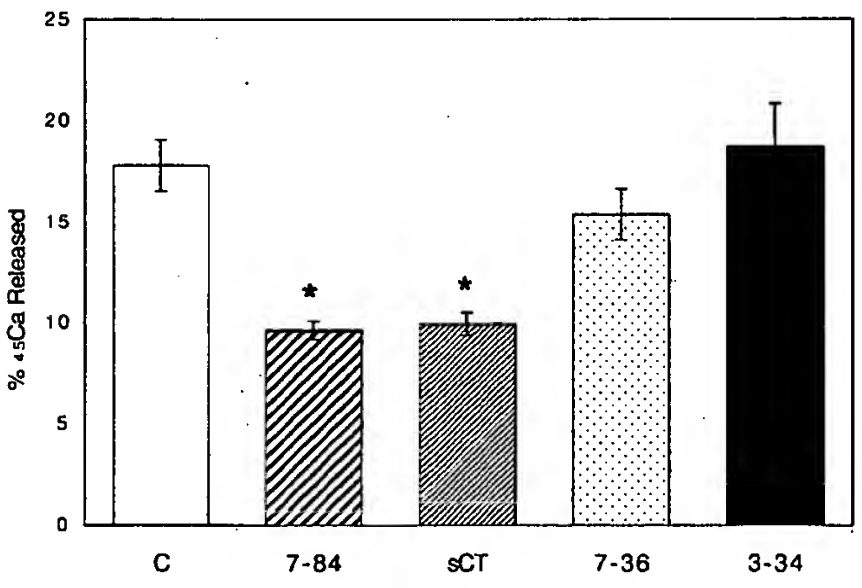

FIG. 1. Inhibition of basal bone resorption by hPTH-(7-84). Calvariae prelabeled with ^{45}Ca by maternal injection were isolated from neonatal mice as described in *Materials and Methods*. Bones were incubated individually, in treatment groups of four bones each, for 72 h after a single addition of vehicle alone (controls, C); hPTH-(7-84), hPTHrP-(7-36), or hPTH-(3-34) (all at 300 nM); or salmon calcitonin (sCT; 100 nM). Results are expressed as percentages of total ^{45}Ca released over 72 h. Values shown are the mean ± SEM of results from several (three to six) independent experiments. *, $P < 0.001$ *vs.* controls.

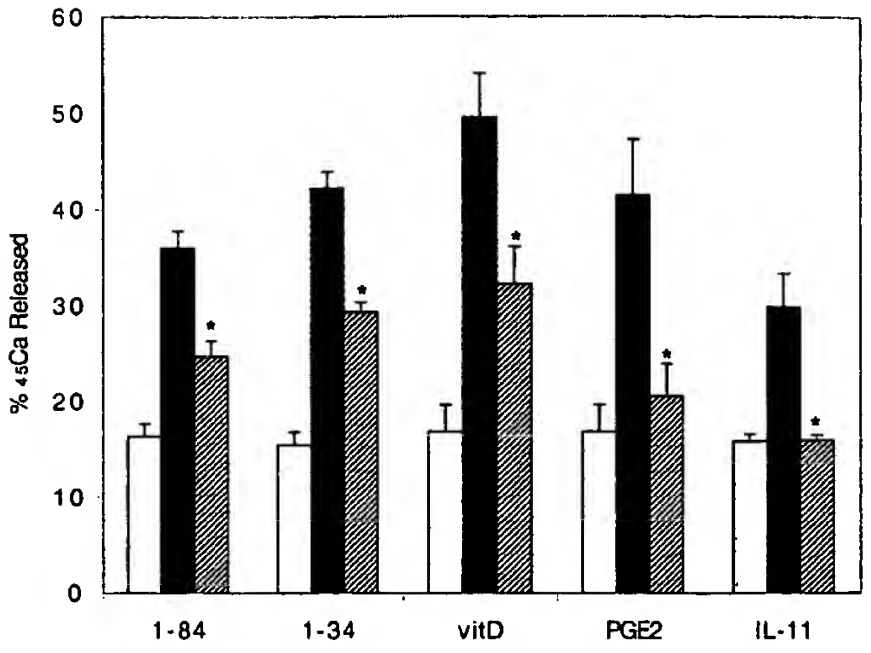

FIG. 2. Inhibition by hPTH-(7-84) of bone resorption induced by various osteotropic agents. Resorption assays were conducted as described in Fig. 1 for controls (□), osteotropic agents alone (■), or osteotropic agents in combination with 300 nM hPTH-(7-84) (▨). Osteotropic agents were employed at the following concentrations: hPTH-(1-84), 3 nM; hPTH-(1-34), 3 nM; VitD, 10 nM; PGE_2, 100 nM; and IL-11, 10 ng/ml. Results are expressed as the mean ± SEM of quadruplicate determinations. Each experiment was repeated three times. *, $P < 0.05$ for difference between osteotropic agent alone *vs.* osteotropic agent plus hPTH-(7-84).

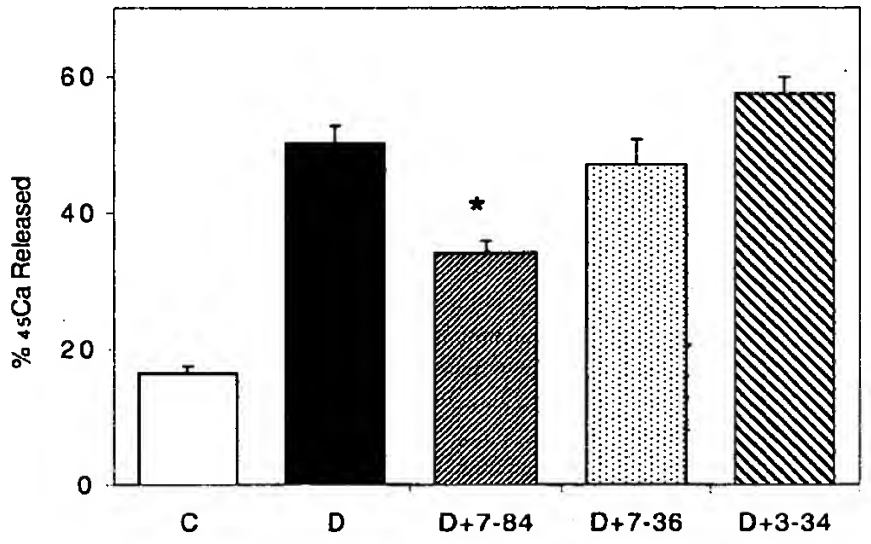

FIG. 3. Inhibition of VitD-induced bone resorption by hPTH-(7-84), but not by hPTHrP-(7-36) or hPTH-(3-34). Calvarial bones, incubated as described in Fig. 1, were treated with vehicle alone (C) or with 10 nM VitD, either alone (D) or together with 300 nM hPTH-(7-84) (D + 7-84), 1 μM hPTHrP-(7-36) (D + 7-36), or 1 μM hPTH-(3-34) (D + 3-34). Results are expressed as the mean ± SEM of quadruplicate determinations and are representative of three independent experiments. *, $P < 0.05$ between VitD alone and VitD plus PTH-(7-84).

resorptive response of the bones to subsequently added VitD (data not shown).

hPTH-(7-84) does not activate adenylyl cyclase or measurably inhibit the binding of a radiolabeled hPTH-(1-34) analog to PTH1Rs expressed on ROS 17/2.8 rat osteosarcoma cells, which also express CPTHRs (21, 27). To directly address the possibility that hPTH-(7-84) nevertheless might antagonize signaling by PTH1R agonists, either directly at the PTH1R or via activation of CPTHRs, ROS 17/2.8 cells were incubated with hPTH-(1-34) at a submaximal concentration (3 nM) in the absence or presence of excess hPTH-(7-84) (0.1–1 μM). As shown in Fig. 4, we observed no inhibition of the cAMP response to PTH-(1-34) by hPTH-(7-84) (0.1–1 μM), whereas both PTHrP-(7-36) and PTH-(3-34), when present at 1 μM, inhibited the response by 50%. In related experiments undiluted samples of conditioned medium from calvarial resorption assays (described above) to which 300 nM hPTH-(7-84), PTHrP-(7-36) or PTH-(3-34) had been added 72 h earlier also were tested for inhibition of hPTH-(1-34)-induced cAMP accumulation in ROS 17/2.8 cells. In none of these samples was inhibition of the cAMP response to hPTH-(1-34) observed (data not shown).

Inhibition of bone resorption by hPTH-(7-84) could result from decreased osteoclast formation, inhibition of the activity or survival of mature osteoclasts, or both. To determine whether hPTH-(7-84) impairs osteoclast formation, the activity of this fragment was studied in cultures of whole murine bone marrow. As shown in Fig. 5A, hPTH-(7-84) alone exerted no effect on the formation of TRAP+MNCs, although basal osteoclast formation in this system is low, and an inhibitory effect therefore might not be easily detectable. On the other hand, when osteoclast formation was stimulated by VitD (10 nM), hPTH-(7-84) (300 nM) caused a striking (70%) reduction in the formation of TRAP+MNCs relative to the effect of VitD alone [VitD, 153 ± 38 cells; VitD + hPTH-(7-84), 53 ± 14 cells]. In contrast,

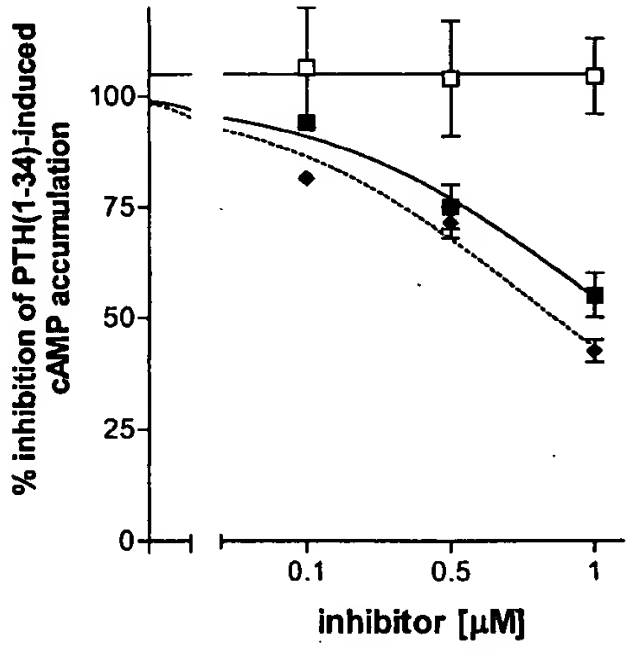

FIG. 4. Inhibition of cAMP accumulation in PTH-treated ROS 17/2.8 cells. Cells were stimulated with an approximately half-maximal concentration of PTH-(1-34) (3 nM) in the absence or presence of increasing concentrations of PTH-(7-84) (□, *solid line*), PTH-(3-34) (◆, *dashed line*), or PTHrP-(7-36) (■, *solid line*). Data are expressed as percentages of the cAMP response to 3 nM PTH-(1-34) alone and represent the results (mean ± SEM) of at least two independent experiments.

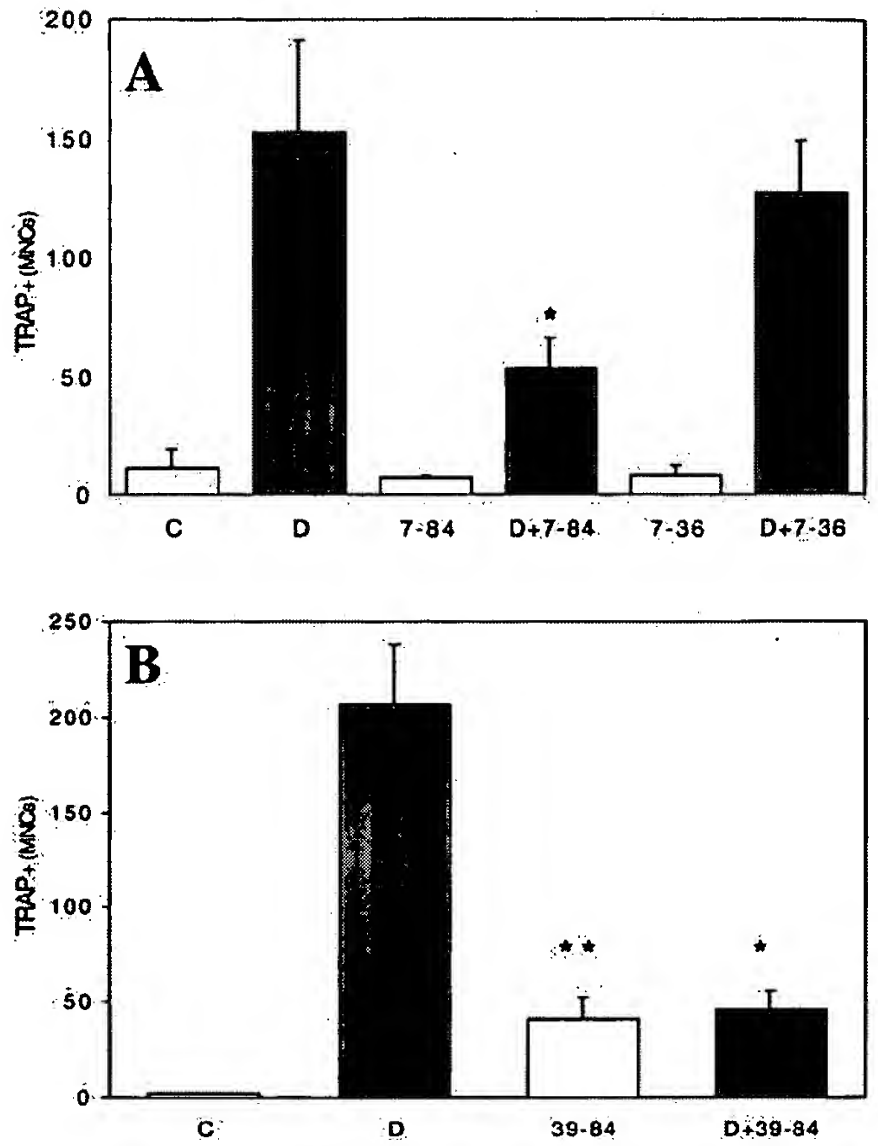

FIG. 5. Inhibition of osteoclast generation by hPTH-(7-84) and hPTH-(39-84). Whole bone marrow was isolated and cultured as described in *Materials and Methods*. Adherent and nonadherent cells were maintained in culture for 12 d, and the indicated treatments were added three times weekly, as described in *Materials and Methods*. At the end of the culture period, cells were fixed and stained for TRAP. A: C, Control; D, VitD (10 nM); 7-84, hPTH-(7-84) (300 nM); 7-36, hPTHrP-(7-36) (300 nM). B: C, Control; D, VitD (10 nM); 39-84, hPTH-(39-84) (3000 nM). Values (number of cells per well) are expressed as the mean ± SD for triplicate determinations (see *Materials and Methods*). Experiments were repeated twice. *, $P < 0.05$ *vs.* VitD alone; **, $P < 0.05$ *vs.* control.

PTHrP-(7-36) did not inhibit TRAP+MNC formation induced by VitD [VitD + PTHrP-(7-36), 127 ± 22 cells; Fig. 5A]. To determine whether shorter C-terminal PTH fragments could regulate osteoclast formation, we tested the effects of hPTH-(39-84), alone or in combination with VitD (10 nM). As shown in Fig. 5B, hPTH-(39-84) alone, at 3000 nM, slightly stimulated osteoclast formation (41 ± 11 cells), as reported previously for short CPTH fragments (24, 33). Like hPTH-(7-84), however, hPTH-(39-84) dramatically inhibited osteoclast formation promoted by VitD [VitD, 207 ± 31 cells; VitD + hPTH-(39-84), 46 ± 10 cells].

Discussion

Recent studies demonstrating that the extended CPTH fragment hPTH-(7-84) can inhibit the calcemic effects of PTH-(1-84) and PTH-(1-34) in thyroparathyroidectomized animals have suggested that CPTH peptides, normally present in blood and previously assumed to be biologically inert prod-

ucts of PTH metabolism, may be physiologically active (20, 21). As the hypocalcemic actions of hPTH-(7-84) described *in vivo* were associated with lowering of serum phosphate but were not accompanied by significant changes in urinary calcium or phosphate excretion (20, 21), a primary effect of this CPTH peptide on bone seems likely. The present *in vitro* studies were directed at clarifying whether hPTH-(7-84) might act directly on bone to inhibit the action of hPTH-(1-84) or hPTH-(1-34).

One possible mechanism for such an effect could involve direct antagonism by hPTH-(7-84) to prevent binding of hPTH-(1-34) or hPTH-(1-84) to PTH1Rs expressed on osteoblasts or marrow stromal cells. Because the antagonism *in vivo* is observed at much lower doses of hPTH-(7-84), relative to intact PTH, than that predicted to be necessary for direct antagonism at the PTH1R, however, we also considered the alternative possibility that hPTH-(7-84) may exert unique PTH1R-independent antiresorptive effects by activating CPTHRs expressed in bone cells. Our results are fully consistent with this latter hypothesis. Thus, we observed concentration-dependent inhibition of bone resorption in *ex vivo* calvarial organ cultures that was not mimicked by shorter, N-truncated PTH fragments that 1) are more effective PTH1R antagonists than is hPTH-(7-84) and 2) do not bind detectably to CPTHRs expressed on bone cells (28). Similar results were obtained in studies of osteoclastogenesis using whole bone marrow cultures, which further suggests that CPTHRs may be involved in the regulation of osteoclast formation. Because the number of mononuclear TRAP-positive cells formed in the marrow cultures also was reduced by hPTH-(7-84), the predominant action in osteoclastogenesis may be to inhibit formation of osteoclast precursors. The rapidity (1–2 h) of the hPTH-(7-84) effect observed *in vivo*, however, suggests that interference with the activity of mature osteoclasts also may be involved.

The antiresorptive effect of hPTH-(7-84) observed in the calvarial assay system contrasted sharply with the inability of hPTH-(3-34) or PTHrP-(7-36), introduced at similar concentrations, to inhibit resorption. Because both of these shorter, N-truncated peptides are effective *in vitro* PTH1R antagonists, whereas hPTH-(7-84) is not (as shown in Fig. 4), these results argue strongly against a mechanism involving direct antagonism by hPTH-(7-84) at the PTH1R of either endogenous PTHrP present within the cultured bones or exogenously added PTH. Moreover, the antiresorptive effect of hPTH-(7-84) *in vitro* was not restricted to resorption induced by added PTH, but was encountered in both control cultures and cultures treated with a variety of unrelated bone-resorbing agonists, including VitD, PGE_2 and IL-11. These findings point to a more generalized antiresorptive mechanism by which PTH-(7-84), presumably acting via CPTHRs, may limit the formation and, possibly, the activity of mature osteoclasts. This could reflect interference with the up-regulation of RANKL or macrophage colony-stimulating factor, the down-regulation of OPG, or both, that normally are triggered in marrow stromal cells and osteoblasts by these diverse resorbing agents (35). In this regard, we observed expression of CPTHRs by PTH1R-null osteoblasts and osteocytes (28) and by clonal marrow stromal cells that are capable of supporting PTH- or vitamin D-dependent osteoclast formation from hemopoietic progenitors *in vitro* (our unpublished observations). We also cannot yet exclude that the inhibition of resorption was mediated partly by a proapoptotic effect of hPTH-(7-84) on bone cells via activation of the CPTHR, as we previously reported in osteocytic cells (28). Moreover hPTH-(7-84) could act directly on mature osteoclasts, their hemopoietic precursors, or both to dampen cellular responsiveness to activation of RANK or c-*Fms* by their respective stromal cell or osteoblast-derived ligands. Indeed, evidence that shorter CPTH fragments alone can modestly induce osteoclast formation, as seen in the present study with hPTH-(39-84) and reported previously (24, 33), in contrast to the inhibitory effects of the same fragment upon osteoclast formation induced by vitamin D, points to a complexity in CPTHR action that is not readily explained at present, but that could involve disparate effects on distinct cell types involved in osteoclast formation. Direct analysis of CPTHR expression in such cells would be needed to address this possibility.

One prediction of our results might be that PTH-(1-84), which binds to CPTHRs with affinity comparable to that of PTH-(7-84) (21), should elicit less bone resorption than PTH-(1-34), which does not interact effectively with CPTHRs (28). Although few direct *in vitro* comparisons have been performed (24, 36), the available data do not indicate substantial or consistent differences in resorptive responses to these two peptides. Indeed, we observed in the calvarial resorption assay that the intact hormone reproducibly induced less ^{45}Ca release than did PTH-(1-34) at equimolar concentrations, although this difference was never statistically significant. This could indicate that when PTH1Rs and CPTHRs are exposed simultaneously to equimolar concentrations of a common ligand, the PTH1R-mediated resorptive response strongly predominates. Alternatively, it is possible that despite comparable binding affinity, intact PTH cannot activate CPTHRs as effectively as N-truncated peptides (by analogy with the disparate activation of PTH1Rs observed with PTH-(1-34) *vs.* PTH-(3-34)). On the other hand, because CPTH fragments normally circulate in plasma at concentrations at least 5- to 10-fold higher than those of intact PTH, a requirement for higher molar concentrations of CPTH ligands to activate CPTHRs might be expected. This concept is consistent with our finding that a 10- to 100-fold molar excess of CPTH ligand is needed to elicit functional antagonism of PTH1R-mediated resorption *in vitro*. The observation that hPTH-(7-84) could antagonize the calcemic response to hPTH-(1-84) at equimolar doses *in vivo* might be related to differential bioavailability or metabolism of the two peptides after their ip or iv administration (20, 21).

Secretion of CPTH fragments by the parathyroid glands is positively regulated by blood calcium (37). Thus, one possible physiological role of the antiresorptive action of N-truncated PTH fragments *in vivo* could be to modulate the extent of bone resorption induced by intact PTH in a manner responsive to the extracellular calcium concentration. Such a mechanism, for example, might allow for maximal release of calcium from bone only during severe hypocalcemia to supplement the ongoing renal and (indirect) intestinal actions of PTH. It is important to note that the chemical identities of all circulating CPTH fragments have not yet been completely defined. In particular, the existence in blood of PTH-(7-84) *per se* has not been directly demonstrated. On the other hand, the

recent immunochemical characterization of nonintact PTH peptides, which are especially abundant in renal failure, is consistent with the presence of extended CPTH fragments longer than those previously inferred from analyses of secreted or peripherally generated cleavage products, the N-termini of which ranged between positions 24 and 43 of the intact PTH sequence (19, 38). Thus, the possibility that PTH fragments similar or identical to PTH-(7-84) may be present in blood, especially in renal failure, at concentrations high enough to activate CPTHRs and thereby exert direct effects on bone resorption must be considered.

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